Regulation of ATM and CDK2 Kinases by the MRN Complex in DNA Damage Signaling and Cell Cycle Checkpoint Control

by

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Dedication

To my parents, Joe and Cynthia Morgan,
And to my husband, David Rogawski,
With all my love.

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List of Abbreviations

APC	Anaphase-promoting complex
A-T	Ataxia-telangiectasia
ATLD	Ataxia-telangiectasia-like disorder
ATLD1	Ataxia-telangiectasia-like disorder 1
ATLD17	Ataxia-telangiectasia-like disorder 17
ASM	Alternative splice mutant
ATM	Ataxia-telangiectasia mutated kinase
ATP	Adenosine triphosphate
ATR	Ataxia-telangiectasia and rad3-related protein
BARD1	BRCA1-associated RING domain protein 1
BER	Base excision repair
BLM	Bloom syndrome protein
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BRCT	BRCA1 C terminus domain
CAK	CDK-activating kinase
CDC25A	M-phase inducer phosphatase 1
CDC45	Cell division control protein 45 homolog
CDC6	Cell division cycle 6

CDC7	Cell division cycle 7-relted protein kinase
CDK	Cyclin-dependent kinase
CDK2	Cyclin-dependent kinase 2
CDT1	DNA replication factor CDT1
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CPT	Camptothecin
CSR	Class switch recombination
CTIP	DNA endonuclease RBBP8
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase, catalytic subunit
DDR	DNA damage response
DSB	Double-strand break
dsDNA	Double-stranded DNA
EXO1	Exonuclease 1
FA	Fanconi Anemia
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GINS	DNA replication complex GINS protein PSF1
HeLa	Henrietta Lacks, human cervical cancer cell line
HR	Homologous recombination
HU	Hydroxyurea

H2AX	H2A histone family, member X
γH2AX	Phosphorylated H2AX
Ig	Immunoglobulin
IgH	Immunoglobin heavy chain
IR	Ionizing radiation
KAP1	KRAB [Kruppel-associated box domain]-associated protein 1
KU70/80	X-ray repair cross complementing 5/6
MCM	Mini chromosome maintenance
MDC1	Mediator of DNA damage checkpoint protein 1
MEF	Mouse embryonic fibroblast
MMC	Mitomycin C
MMEJ	Microhomology-mediated end-joining
MRE11	Meiotic recombination 11-like protein A
MRN	Mre11/Rad50/Nbs1 complex
MTOR	Mechanistic target of rapamycin kinase
MYC	Myc proto-oncogene protein
MYT1	Membrane-associated tyrosine-and-threonine-specific cdc2-inhibitory kinase
NBS	Nijmegen breakage syndrome
NBS1	Nijmegen breakage syndrome 1
NHEJ	Non-homologous end joining
ORC	Origin recognition complex
PALB2	Partner and localizer of BRCA2

PARP1	Poly [ADP-ribose] polymerase 1
PI	Propidium Iodine
PIKK	Phosphatidylinositol 3-kinase-related kinases
P53	Cellular tumor antigen p53
RAD50	DNA repair protein RAD50
RAD51	RAD51 recombinase
RAG1	Recombination activating gene 1
RAG2	Recombination activating gene 2
RB	Retinoblastoma protein
RDS	Radioresistant DNA synthesis
RNF8	Ring finger protein 8, E3 ubiquitin ligase
ROS	Reactive oxygen species
RPA	Replication protein A
RSS	Recombination signal sequences
SCF	Skp, Cullin, F-box containing complex
SCID	Severe combined immunodeficiency
SHM	Somatic hypermutation
SKP2	S-phase kinase associated protein 2
SMC1	Structural maintenance of chromosome protein 1
ssDNA	Single-stranded DNA
TOPO1	DNA topoisomerase 1
UV	Ultra-violet
U2OS	Human bone osteosarcoma epithelial cells

WEE1	Wee1-like protein kinase
XLF	Non-homologous end-joining factor 1
XRCC1	X-ray repair cross-complementing protein 1
XRCC4	X-ray repair cross-complementing protein 4
4-OHT	4-hydroxytamoxifen
53BP1	Tumor suppressor p53-binding protein 1

Abstract

Double-stranded breaks (DSBs) are toxic DNA lesions that if left unrepaired, lead to mutations, chromosomal aberrations, and oncogenesis. The DNA Damage Response (DDR) is a complex network of signaling pathways designed to detect and repair DNA breaks and thus acts as a critical anti-cancer barrier. The MRN complex, consisting of the proteins MRE11, RAD50, and NBS1, is a sensor of DSBs and has roles in facilitating activation of the ataxia-telangiectasia mutated (ATM) kinase, the master regulator of the DDR pathway. ATM phosphorylates a variety of proteins involved in initiating cellular responses important for safeguarding the genome, such as DNA repair, cell cycle checkpoints, and if the damage is too catastrophic to be fixed, apoptosis. A second DNA DSB sensor exists called the KU70/KU80 heterodimer, which binds and activates the kinase DNA-dependent protein kinase, catalytic subunit (DNA-PKcs).

To investigate the apparently redundant roles of MRN and KU70/80 in DNA damage kinase signaling, we utilized cells derived from mouse models deficient in either MRN, KU, or both in conjunction with pharmacologic inhibitors for the respective kinases of each DNA damage sensor, ATM and DNA-PKcs. We found that when MRN is deficient, DNA-PKcs effectively substitutes for ATM. Surprisingly, in the absence of both MRN and KU, ATM is still recruited to the chromatin and can facilitate local chromatin responses, including phosphorylation

of the histone variant H2AX and recruitment of MDC1. This data implies that MRN is not absolutely required for activation of ATM, as previously thought.

Our lab has previously described that the MRE11 C-terminus interacts with cyclin-dependent kinase 2 (CDK2), a kinase important for S-phase cell cycle progression, and that this interaction has roles for homology-directed DSB repair in normally dividing cells. Interestingly, the MRE11 C-terminus is absent in an inherited patient allele causing ataxia-telangiectasia-like disorder 1 (ATLD1). To further understand roles of the MRE11-CDK2 interaction in the DDR, we treated cells with ionizing radiation (IR) to induce DSBs. We found that DNA damage disrupts this interaction in an ATM-dependent manner and causes a reduction in CDK2 catalytic activity. Next, we found that genetic disruption of the interaction in cells lacking either the MRN complex or the MRE11 C-terminus also led to decreased levels of CDK2 activity, mimicking IR-induced disruption of the interaction. Taken together, our data reveal a novel pathway of S-phase checkpoint regulation by the MRE11-CDK2 interaction.

To gain greater insight into roles of the MRN complex in cancer, we engineered a mouse model with B lymphocytes lacking MRN or MRN-nuclease activities. Both forms of MRN deficiency led to characteristics of cancer, including oncogenic translocations between C-MYC and the immunoglobulin locus. Surprisingly, these B lymphocytes did not progress to detectable B lineage lymphoma, even in the absence of p53. Moreover, MRE11-deficiencies prevented tumorigenesis in a mouse model strongly predisposed to spontaneous B-cell lymphomas. Our findings indicate that MRN is not a classic tumor suppressor and

instead imply that certain functions of the MRN complex, such as MRE11 nuclease activity, are required for oncogenesis. These data also suggest that MRE11 nuclease activity can be targeted for cancer therapy development.

Chapter I

Introduction

The Hallmarks of Cancer

Cancer is a leading cause of death in people of all ages in the United States and around the world. According to the American Cancer Society, about half of all men and one-third of all women in the US will develop cancer during their lifetimes. In 2000, Douglas Hanahan and Robert Weinberg proposed six fundamental hallmarks of cancer[1]. Since then, much progress has been made in understanding the complexities of cancer and additional hallmarks have been identified[2]. Two important hallmarks, evading growth suppressors and genomic instability/mutation, lie at the heart of my thesis work (Figure 1.1).

One of the most fundamental traits of cancer cells is their ability to proliferate indefinitely[1, 2]. Normal cells carefully control the production and release of growth-promoting signals that control entry and progression through the cell cycle, while cancer cells deregulate these signals. Uncontrolled growth of cancer cells in part leads to tumor formation[1, 2]. Additionally, DNA damage response mechanisms are essential in maintaining genomic stability and preventing chromosomal translocations and mutations. Defects in genome maintenance and repair are selectively advantageous and instrumental for tumor progression[1, 2]. Thus, a better understanding of the cell cycle machinery that

controls cell growth and the DNA repair machinery that maintains stability of the genome are critical not only in deciphering mechanisms of neoplastic transformation, but also for the development of anti-cancer drugs and treatments.

The DNA Damage Response

Sources and types of DNA Damage

The DNA in our cells is damaged every day. These lesions are dangerous because they can block important processes such as replication and transcription, and if not properly repaired, can lead to mutations and chromosomal aberrations that threaten the survival of both cells and organisms[3].

There are numerous types of DNA damage caused by a varying array of sources. Lesions can be spontaneous, induced endogenously, by radiation, or by chemicals[4]. DNA lesions can arise from endogenous physiological processes such as during the replication process and by reactive oxygen species that arise as by-products from oxidative respiration[5]. One of the most prominent sources of environmental damage to DNA is ultra-violet light (UV light), which can induce ~100,000 lesions per exposed cell per hour[6]. Ionizing radiation (IR) also generates various forms of DNA damage, the most toxic of which is DNA double-strand breaks (DSBs)[7]. Some ionizing radiation results from radioactive decay of naturally occurring radioactive compounds. Exposure to natural or man-made radioisotopes also occurs during cancer radiotherapy[8]. Chemicals that are known to cause mutations and cancer are known as mutagens or

carcinogens. Examples of carcinogens include: Mitomycin C (MMC), an alkylating agent that inhibits DNA synthesis by covalently reacting with DNA, forming crosslinks; Aphidicolin, a specific inhibitor of DNA polymerase α; hydroxyurea, an inhibitor of DNA synthesis; Camptothecin (CPT), an inhibitor of the DNA enzyme topoisomerase I; and Bleomycin, which induces oxidative stress and DNA damage via single-strand breaks (SSBs) and DSBs[5, 9-12].

DSBs are among the most toxic DNA lesions and result from either endogenous or exogenous sources[13]. Naturally occurring DSBs are generated spontaneously during DNA synthesis when the replication fork encounters a damaged template and during specialized processes of immune development, including V(D)J recombination and class switch recombination (CSR)[14]. Programmed DSBs during meiosis are essential for the exchange of genetic information between homologous chromosomes and for correct chromosome segregation[3]. Environmental sources of DSBs include ionizing radiation (X-rays and gamma rays), topoisomerase poisons, and radiometric drugs[15].

DNA DSB repair pathways

Cells have evolved complex signaling mechanisms, together known as the "DNA damage response" (DDR), to detect, signal, and repair DNA breaks (Figure 1.2). Cells defective in DDR mechanisms often exhibit hypersensitivity to DNA damaging agents, and many defects in the DDR can lead to human disease[3]. The diverse range of DNA lesion types requires multiple, distinct DNA

repair mechanisms[16]. In mismatch repair, detection of mismatches triggers a single-strand incision that is then acted upon by nuclease, polymerase, and ligase enzymes[17]. In base-excision repair, a damaged base is often recognized by a DNA glycosylase enzyme that mediates removal of the base[18]. During nucleotide excision repair (NER), the damage is excised as a 22-30 base oligonucleotide, producing single-stranded DNA (ssDNA) that is acted upon by DNA polymerases and associated factors before being ligated[19, 20]. For DSB repair, two principle methods are used: non-homologous end joining (NHEJ) and homologous recombination (HR) (Figure 1.3).

Non-homologous end-joining

NHEJ is a “cut and paste” method of DSB repair. In NHEJ, DSBs are recognized by the KU protein (a heterodimeric complex consisting of KU70 and KU80 proteins) that binds and activates the protein kinase DNA-dependent protein kinase, catalytic subunit (DNA-PKcs)[21, 22]. Since DSBs can occur with a variety of different ends, several processing enzymes are required to fix the breaks. ARTEMIS, a member of the metallo-β-lactamase superfamily of enzymes, has both a DNA-PKcs-independent 5’-to-3’ exonuclease activity towards DNA-containing ds-ssDNA transitions and DNA hairpins, each of which might be important for processing of DNA termini during NHEJ[23]. Repair by NHEJ is completed by ligation of the DNA ends, which is carried out by X-ray repair cross-complementing protein 4 (XRCC4) and DNA ligase IV[24-26]. A less well-characterized KU-independent NHEJ pathway, known as microhomology-

mediated end-joining (MMEJ) or alternative end-joining, also exists and results in sequence joins[27]. Both NHEJ and MMEJ are error-prone but can operate in any phase of the cell cycle. Core members of the NHEJ pathway perform direct roles in ligating the programmed DSBs produced during both V(D)J recombination and CSR[28-30]. NHEJ is inhibited by end-resection (HR), the process by which 5'-3' nucleolytic degradation generates ssDNA.

Homologous recombination

Repair by HR is generally error-free and restricted to the S and G2 phases of the cell cycle when sister chromatids are more easily accessible following replication. The MRN complex, a sensor of DSBs, is required for the recruitment and activation of the ataxia-telangiectasia mutated (ATM) kinase[31-34]. HR involves processing of the DSB to yield a 3' ssDNA overhang. It is thought that the nuclease activities of MRE11, along with DNA endonuclease RBBP8 (CTIP), collaborate to resect the DNA ends to an intermediate form[35-37]. The DNA ends are further processed into ssDNA by exonuclease 1 (EXO1) and Bloom syndrome protein (BLM) helicase[38-40]. The ssDNA is then rapidly bound by the ssDNA-binding protein termed replication protein A (RPA) which melts the DNA's secondary structure[41]. A mediator complex containing breast cancer type 1 susceptibility proteins 1 and 2 (BRCA1/2), BRCA1-associated RING domain protein 1 (BARD1), and partner and localizer of BRCA2 (PALB2) catalyze the nucleation of RAD51 recombinase (RAD51) onto the free 5' end of a dsDNA-ssDNA junction, such that RPA is replaced by RAD51 to form a

nucleofilament[42]. The RAD51 nucleoprotein filament captures duplex DNA and searches for homology sequences (usually a sister chromatid) which is followed by strand invasion and finally resolution[42-44]. The critical role of HR in suppressing genomic instability is reflected in the early embryonic lethality of mice lacking *Rad51*, *Brca1*, and *Brca2*[45-47].

Chromatin response in DSB repair

Chromosomal DSBs in eukaryotes exhibit a rapid and extensive response in the chromatin flanking the break, highlighted by the phosphorylation of the histone H2AX in mammalian cells (denoted as γH2AX)[48]. The H2AX phosphorylation site, Serine 139, is a common recognition site for the phosphatidylinositol 3-kinase-related kinases (PIKK) family members, including ATM, ataxia-telangiectasia mutated- and Rad3-related (ATR), and DNA-PKcs[48-51]. All have the potential to phosphorylate H2AX, but ATM appears to be the main kinase associated with γH2AX formation under normal physiological conditions[34, 50]. The γH2AX mark around a DSB can extend more than 1 megabase from the break[52]. Many DNA damage response proteins, such as Mediator of DNA damage checkpoint protein 1 (MDC1), the MRN complex, ATM, p53-binding protein 1 (53BP1) and BRCA1/BARD1, accumulate on γH2AX-containing chromatin[53-56]. MDC1 is the critical adaptor protein that directly interacts with γH2AX[56]. Interestingly, BRCA1 and MRN/ATM can also associate with DSBs in $H2AX^{-/-}$ cells, suggesting γH2AX-independent roles at the DSB[57]. The recruitment of 53BP1 and BRCA1 to γH2AX chromatin is indirect,

requiring the activity of the E3 ubiquitin ligases RNF8 and RNF168[58, 59]. Thus, it is clear that chromatin changes after DNA damage are essential for the recruitment of DDR factors and other chromatin-modifying components, which together, are thought to promote DSB repair and amplify DSB signaling[52, 60].

DNA Damage Response signaling

The DNA damage response is a complex network of signaling pathways designed to detect, signal, and repair DNA lesions. The presence of a DNA lesion is recognized by sensor proteins, allowing for recruitment of mediator proteins, transducers and effectors, and initiation of cellular responses such as transcription, cell cycle checkpoints, chromatin remodeling, DNA repair, and apoptosis/senescence[3]. The master regulators of the signaling network are the protein kinases ATM and ATR. ATM is recruited and activated by DSBs, whereas ATR is recruited and activated by RPA-coated ssDNA[31, 41, 61]. The two primary targets of ATM and ATR are checkpoint kinases 1 and 2 (CHK2 and CHK1), respectively[62-64]. CHK2 and CHK1, together with ATM and ATR, act to reduce cyclin-dependent kinase (CDK) activity by various mechanisms[62]. Inhibition of the CDKs slows down or arrests cell cycle progression at the G1-S, intra-S, and, G2-M cell cycle checkpoints, which allows for DNA repair to occur before either replication or mitosis begins[65, 66]. Additionally, ATM and ATR enhance repair by inducing the transcription of DNA repair proteins, by recruiting repair factors to the damage, and by activating DNA repair proteins by modulating their phosphorylation, acetylation, ubiquitylation, or SUMOylation[67, 68]. If the above

events allow for effective DNA repair, inactivation of the DDR occurs, allowing for resurgence of normal cell functioning[3]. Alternatively, if the damage cannot be fixed, chronic DDR signaling triggers cell death by apoptosis or cellular senescence (permanent removal from the cell cycle)[69-72].

The DDR and human disease

Genomic instability is a fundamental hallmark of cancer[1, 2]. In lymphoid tumors, genomic instability frequently corresponds to chromosomal translocations[73]. These translocations result when proto-oncogene loci fuse together with antigen receptors by an aberrant antigen-receptor recombination mechanism[74]. Defects in mismatch repair can cause microsatellite instability which is common in colorectal and endometrial carcinomas[17]. Additionally, inherited defects in the DDR machinery commonly predispose patients to cancer[3]. Thus, problems in the DDR arising from mutational or epigenetic inactivation of DDR components allow for malignant progression. Therefore, the DDR is often viewed as an important barrier to cancer.

Accumulation of DNA lesions in neurons is associated with neurodegenerative disorders such as Alzheimer's, Huntington's and Parkinson's diseases[75]. Furthermore, there are roughly 40 known diseases that result from expansions or contractions of genetic unstably DNA repeat sequences[75]. These neuromuscular and neurodegenerative diseases include fragile X syndromes, Friedrich's ataxia, diabetes mellitus type 2, and Huntington's disease[75-77].

Genomic rearrangements involving DDR factors occur during immune-system development resulting in immunodeficiency[78]. For example, patients with mutations in NHEJ factors are often B- and T-cell immune deficient, whereas ataxia-telangiectasia (A-T) and Nijmegen breakage syndrome (NBS) patients are prone to sometimes fatal infections due to an impaired immune system[79, 80]. Furthermore, many lymphomas and leukemias of B- and T-cell origin arise from impaired V(D)J recombination[74]. Because meiotic recombination involves DSB generation, certain DDR defects can cause human infertility[81, 82]. Finally, there is evidence that aging is in part caused by accumulated DNA damage[83]. With this knowledge, it makes sense that studying the DDR can elucidate ways in which we can treat cancer and human diseases.

Kinases of the DNA Damage Response

Ataxia-telangiectasia mutated kinase

ATM is the central kinase of the DNA damage response. ATM is a serine-threonine kinase that is activated in response to DNA DSBs and is a member of the PIKK family of serine/threonine kinases, which also includes DNA-PKcs, ATR, and mechanistic target of rapamycin kinase (MTOR) kinases[31, 84, 85]. ATM phosphorylates a variety of proteins involved in DNA repair, cell cycle checkpoint control, and apoptotic responses, including CHK2, p53, BRCA1, H2AX, structural maintenance of chromosome protein 1 (SMC1), ARTEMIS, and NBS1[48, 86-91]. Phosphorylation of these and other substrates by ATM initiates cell cycle

arrest at G1/S, intra-S, and G2/M checkpoints and also promotes DNA repair[92]. Mutations in the *ATM* gene are responsible for the rare autosomal recessive disorder ataxia-telangiectasia, which is characterized by cerebellar degeneration, immunodeficiency, and an increased risk of cancer[93]. Cells from individuals with A-T exhibit defects in DNA damage-induced checkpoint activation, radiation hypersensitivity, and an increased frequency of chromosomal aberrations[94].

The functional relationship between ATM and the MRN complex was initially discovered when it was found that patients with similar clinical phenotypes observed in AT patients had mutations in *MRE11* (A-T like disorder or ATLD)[95, 96]. Loss of any of the individual components of the MRN complex results in embryonic lethality in mice[97-99]. ATLD patients express low levels of mutant MRN protein, so ATLD most likely represents MRN hypomorphism rather than complete loss of the complex[100]. Interestingly, ATLD cells with mutant MRE11 have markedly reduced activation of ATM by DNA DSBs and reduced ATM-dependent phosphorylation of downstream substrates, demonstrating that MRN is required for optimal ATM activation following DNA DSB induction[96, 100]. The work in Chapter II expands upon and updates previous models for MRN's requirement in ATM activation.

ATM is mostly a nuclear protein, where it exists as an inactive homodimer. DNA damage, however, converts the inactive homodimer to an active monomer form that phosphorylates a large number of substrates[101, 102]. Additionally, ATM autophosphorylation occurs at Serine 1981 at the same

time as monomerization/activation of ATM in response to DNA damage[101]. The transformation of inactive ATM into an active kinase has been observed with purified proteins, Xenopus egg extracts, and human cell extracts *in vitro*[31, 103, 104]. In addition to the presence of DNA DSBs, there are reports that ATM can be activated by oxidative stress or reactive oxygen species[105].

One murine model of ataxia-telangiectasia was created by disrupting the *Atm* locus via gene targeting[106]. Mice homozygous for the disrupted *Atm* allele are viable, but display growth retardation, neurologic dysfunction, male and female infertility, defects in T-lymphocyte maturation, and sensitivity to radiation[106]. The majority of mice developed malignant thymic lymphomas and several chromosomal abnormalities were detected in the tumors[106]. Fibroblasts from these mice grow slowly and exhibit abnormal G1 checkpoint function[106]. Thus, *Atm*-deficient mice recapitulate the ataxia-telangiectasia phenotype in humans and provide a mammalian model to further study the disease[106].

ATM is a known tumor suppressor frequently mutated in a broad range of human cancers[107]. Along with its tumor suppressor capabilities, the hypersensitivity of ATM-defective cells to IR has sparked interest in ATM as a therapeutic target for cancer therapy. Early studies demonstrated that caffeine inhibits the catalytic functions of both ATM and ATR[108, 109]. KuDOS Pharmaceuticals (acquired by AstraZeneca in 2005) developed the first potent and selective ATM inhibitor: KU-55933. KU-55933 confers marked sensitization to IR and DNA DSB-inducing chemotherapeutics, such as the topoisomerase II

inhibitors etoposide and doxorubicin, in cancer cells. Importantly, in cells derived from A-T patients, which express no functional ATM, no radiosensitization was observed, further confirming the selectivity of the compound for ATM[110]. Even though no ATM inhibitors are in clinical development yet, the *in vitro* studies carried out to date clearly show that pharmacological ATM inhibition has great potential as a cancer therapy in combination with radiotherapy or certain chemotherapeutic drugs.

DNA-dependent protein kinase, catalytic subunit kinase

DNA-PKcs is a member of the PIKK family of kinases[111]. DNA-PKcs is required for NHEJ and V(D)J recombination. Cells lacking DNA-PKcs are highly radiosensitive and have defects in V(D)J recombination[112]. *DNA-PKcs* deficiency in mice is associated with severe combined immunodeficiency (SCID)[112, 113]. Importantly, DNA-PKcs interacts with the DNA DSB sensor, KU[21]. KU and DNA-PKcs only interact in the presence of DNA and recruitment of DNA-PKcs to sites of DNA damage *in vivo* is KU-dependent[21]. DNA-PKcs has serine/threonine kinase activity and phosphorylates a number of substrates. The protein kinase activity of DNA-PKcs is required for NHEJ, and inhibitors of DNA-PKcs kinase activity radiosensitize cells and inhibit DSB repair, making DNA-PK a possible therapeutic target.

Ataxia-telangiectasia and rad3-related protein kinase

ATR is another member of the PIKK family of kinases. ATR is essential for the viability of replicating human and mouse cells and is activated in S-phase to regulate the firing of replication origins and the repair of damaged replication forks[114]. Mutations in *ATR* are rare and the only current link between *ATR* gene mutation and disease exists in a few patients with the rare Seckel Syndrome, which is characterized by growth retardation and microcephaly[115]. Disruptions in the ATR pathway cause genomic instability. ATR is activated in response to persistent ssDNA, which is a common intermediate formed during DNA damage detection and repair[116, 117]. Once ATR is activated, it phosphorylates CHK1, initiating a signal transduction cascade that culminates in cell cycle arrest[63, 118]. In addition to its role in activating the DNA damage checkpoint response, ATR is thought to function in unperturbed DNA replication[119]. *Atr* heterozygous mice develop normally and demonstrate a significantly increased tumor risk by 18 months of age, while homozygous *Atr* mice exhibit high numbers of chromosomal breaks, proliferation defects in cell culture, and early embryonic lethality[120-122].

Sensors of DNA double-strand breaks

The MRE11/RAD50/NBS1 complex

The MRN complex is a multi-protein complex consisting of meiotic recombination 11 (MRE11), DNA repair protein RAD50, and Nijmegen breakage syndrome 1 (NBS1) proteins[123]. The MRN complex is an important player in the

DDR, as it senses and physically binds to areas of DNA damage rapidly after insult, tethers and bridges the broken DNA ends together over both long and short distances, and is involved in activating pathways of DNA repair and cell cycle checkpoints[123, 124]. Importantly, MRE11 and RAD50 are conserved throughout evolution across all domains of life, whereas NBS1 is specifically found in eukaryotes and is less well conserved[123]. Another significant function of MRN is its involvement in activation of the ATM signaling pathway. Activated ATM phosphorylates a variety of proteins involved in DNA repair, cell cycle checkpoint control, and apoptosis[31, 32, 125, 126] (Figure 1.4).

MRE11

MRE11 is a nuclear protein that has both exo- and endo-nuclease activities. This includes dsDNA 3' to 5' exo-nuclease activity, as well as ssDNA endo-nucleolytic activity[39, 127-129]. The N-terminus of MRE11 has four highly conserved nuclease domains as well as a phosphodiesterase domain[130]. The third nuclease domain contains a highly conserved histidine residue (H129) that has been shown by both *in vitro* and structural studies to act directly in DNA catalysis by stabilizing the sugar-phosphate moiety during nucleolysis[130-132]. Surprisingly, in yeast, mutation of this histidine residue causes only a mild radiation hypersensitivity phenotype[133, 134]. More recently, our lab has shown that MRE11-nuclease deficiency by mutation of the H129 residue causes a striking array of phenotypes in mice, including early embryonic lethality and severe genomic instability[135]. The nuclease activities also have important roles in

homology-directed DNA repair and roles in activating the ATR kinase[129, 136-138]. MRE11 functions as a dimer to bind both sides of a DSB and stabilize them in close proximity to each other. The nucleolytic activities of MRE11 can then go on to process the bound ends once inside the dimer[139]. The MRE11 C-terminus is more disordered and less conserved than the N-terminus, and contains the DNA-binding domain in budding yeast[123]. The C-terminal domain is the portion of MRE11 mutated in a distinct patient allele that causes ataxia-telangiectasia-like disorder 1 (ATLD1)[100]. Our lab discovered that the C-terminal portion of MRE11 is required for its interaction with cyclin dependent kinase 2 (CDK2)[140]. I describe roles for the MRE11-CDK2 interaction in the DNA damage response in Chapter III of this thesis.

RAD50

RAD50 is another member of the MRN complex, which has both ATPase and adenylate kinase functions[141, 142]. The protein encoded by this gene is highly similar to yeast Rad50, which also functions in DSB repair[143]. RAD50 protein contains a long internal coiled-coil domain that folds back on itself, bringing the N- and C-termini together to form a globular ATPase head domain[144, 145]. MRE11 and RAD50 form a heterotetramer together that functions as the DNA binding and processing core of the complex[146, 147]. *Rad50*-null mice are embryonic lethal, while hypomorphic *Rad50*-mutant mice exhibit growth defects, cancer predisposition, and hematopoietic failure[97, 148].

NBS1

NBS1 is less well-conserved than the other members of the complex, but has a yeast homolog known as *Xrs2*[149]. The NBS1 protein contains three functional regions: the forkhead-associated (FHA) domain and BRCA1 C-terminus (BRCT) domain at the N-terminus, several SQ motifs (consensus phosphorylation sites by ATM and ATR kinases) in the central region, and an MRE11-binding region on the C-terminus[150, 151]. Previously, it was thought that NBS1 provides the MRN complex with its signaling role by binding to and activating ATM in response to DNA DSBs, however, our lab has recently shown that this interaction is not absolutely required for activation of ATM[34, 152]. Mutations in *NBS1* are associated with Nijmegen breakage syndrome (NBS), a radiation-hypersensitivity disease[153]. Characteristics of NBS include microcephaly, growth retardation, immunodeficiency, and a predisposition to cancer[154]. Though *Nbs1*-null mice are not viable, mice with *Nbs1* heterozygosity or hypomorphism are predisposed to oncogenesis[98, 125].

Ataxia-Telangiectasia-Like Disorder

Mutations in *MRE11* cause the disease known as ataxia telangiectasia like-disorder (ATLD)[100]. ATLD is a rare autosomal recessive disorder characterized clinically by progressive cerebellar degeneration resulting in ataxia[100]. Patient cells have low levels of the MRN complex, defects in cell cycle checkpoint activation, hypersensitivity to radiation, and an increase in chromosomal aberrations as compared to controls, all consistent with a defect in DNA repair[96,

100]. The disorder shares some phenotypic features of A-T, but telangiectasia and immunodeficiency are not present in ATLD. Patients have a varied predisposition to cancer[100].

To date, several ATLD patient alleles have been identified. *MRE11*^{ATLD1} was the first reported ATLD allele and contains a nonsense mutation that truncates 76 amino acid residues from the C-terminus of MRE11. *MRE11*^{ATLD1} patients and mice exhibit genomic instability, but do not appear to be prone to cancer[100]. The *MRE11*^{ATLD17} mutation results in substitution at a highly conserved residue in the N-terminal phosphodiesterase domain[155]. *MRE11*^{ASM} mutation causes skipping of exon 10 of MRE11. Two brothers with *MRE11*^{ATLD17} and *MRE11*^{ASM} mutations died prematurely of pulmonary adenocarcinoma, while the brothers' heterozygous family members did not have ATLD or lung cancer[156].

In addition to inherited mutations causing ATLD, *MRE11* has been found to be mutated in both inherited and sporadic cancers. Mutational analysis of *MRE11* in primary tumors has revealed a heterozygous mutation encoding *MRE11* R572Q in a lymphoma[157]. The mutation resides in an interesting region of *MRE11* known as a glycine-arginine-rich (GAR) motif which has been shown to be important for MRE11 exonuclease activity and activation of ATR[137]. Alterations of the GAR motif arginines in mice cause radiation hypersensitivity, a trait common among cancer-prone genomic instability syndromes[137, 158-160]. Studies from our lab have determined that the varied spectrum of disease-associated *MRE11* mutations impact DNA damage signaling and disease outcomes by distinct mechanisms[161] (Figure 1.5).

Mouse and cellular models of MRE11

We have a better understanding of the functions and roles of MRN in large part due to the generation of mouse models of the complex. As stated previously, knockout alleles of *Mre11*, *Rad50*, and *Nbs1* are all embryonic lethal in mice[97, 99, 162]. Interestingly, *Mre11*-nuclease deficiency in mice is also embryonic lethal[163]. Embryonic lethality, thus, is an issue that must be overcome to fully understand the biological functions of MRE11. To bypass the embryonic lethality conferred by *Mre11* deficiencies, our lab disabled the MRN complex through use of mouse embryonic fibroblasts (MEFs) that originally contained one *Mre11*-null allele and one Cre/LoxP conditional allele[135]. Exposure to Cre recombinase via adenovirus converts these cells to MRE11-null cells which also causes deficiency of RAD50 and NBS1[163]. Buis et al., studied *Mre11*-nuclease deficiency by mutating a conserved histidine residue in the nuclease domain to an arginine (H129N). MRE11-null MEFs display growth retardation, defects in ATM activation and the G2/M checkpoint, and an increase in chromosomal abnormalities[135]. On the other hand, MRE11-nuclease deficient MEFs display intact ATM activation, but have a defect in DNA repair and are hypersensitive to IR similar to MRE11-deficient cells[163]. These findings suggest that nucleolytic processing by MRE11 is an essential function of fundamental importance in DNA repair, distinct from MRN control of ATM signaling.

Mice expressing one of the *MRE11* alleles inherited in ATLD were recently derived[95]. The mutation, which causes truncation of the MRE11 C-terminus, had a profound effect on maternal embryonic viability, revealing a requirement for the

MRN complex in early embryogenesis. Cells derived from *Mre11*$^{ATLD1/ATLD1}$ mice had low levels of the MRN complex, impaired ATM signaling, marked chromosomal instability, but no predisposition to malignancy[164]. However, low levels of the MRN complex in ATLD-associated mutations prevent structure-function analyses through study of patient cells lines. To overcome this limitation, our lab previously engineered cell lines that express MRE11 mutants of interest at approximately physiologic levels and in which endogenous wild-type MRE11 can be inactivated through addition of adeno-cre virus *in vitro[165]*. Surprisingly, expression of *Mre11*^{ATLD1} at physiologic levels in mouse cells rescued ATM signaling and G2/M checkpoint activation[165]. This data provides evidence that reduced ATM signaling in *MRE11*^{ATLD1} patients likely results primarily from low levels of the MRN complex since expression of *Mre11*^{ATLD1} to normal levels in mouse cells supports ATM activity.

Post-translational modifications of the MRN complex

The components of the MRN complex are phosphorylated by PIKK enzymes and other kinases, with varying effects on DNA repair as well as on ATM signaling[166]. MRE11 is phosphorylated on at least five C-terminal SQ/TQ sites by ATM and ATR following DNA damage, and these modifications reduce the affinity of MRN for DNA, resulting in lower levels of ATM recruited to damage sites[167]. RAD50 is also phosphorylated by ATM[88]. NBS1 is phosphorylated by ATM on Serines 278 and 343, modifications that are required for S-phase checkpoint control and radiation-induced DNA damage

responses[168]. Furthermore, several studies have shown that NBS1 is a target of CDK phosphorylation[169, 170]. NBS1 Serine 432 phosphorylation occurs in S, G2, and M phases of the cell cycle and requires CDK activity. Data indicates that CDK-dependent NBS1 Serine 432 phosphorylation is critical for promoting MRN-dependent resection and HR and is also needed for MRN-dependent recovery from replication arrest[169, 171]. Finally, a recent study showed that NBS1 is a target of K63-linked ubiquitination by the E3 ligase S-phase kinase associated protein 2 (SKP2) and that this modification affects ATM autophosphorylation, recruitment to chromatin via MRN interaction, and ATM substrate phosphorylation[172].

Roles for MRN in lymphoid malignancy

Programmed DSBs occur during V(D)J recombination, class switch recombination, and somatic hypermutation (SHM), important processes for the development of the adaptive immune system in all vertebrates[173]. These processes occur in developing B and T lymphocytes to generate immunoglobulin (Ig) and T-cell receptor (TCR) diversity, which allows for effective recognition of diverse pathogens and antigens[174]. During V(D)J recombination, exons encoding the V, D, and J segments are combined in various ways to form mature Ig and TCR genes. Each segment is flanked by recombination-signal sequences (RSS) that are recognized by the recombination activating gene (RAG) protein complex. This complex creates a blunt DSB at the signal sequence and a DNA hairpin at the coding end. These structures are then processed and ligated by NHEJ repair mechanisms[174].

Lymphoid malignancies are cancers that affect the blood, bone marrow, lymph, and lymphatic system. Although present in non-lymphoid tumors, chromosomal translocations represent the hallmark of lymphoid malignancies[78]. One example of a common translocation is found in Burkitt's lymphoma[175]. This cancer carries a reciprocal translocation that results in the fusion of the coding region of the proto-oncogene protein (C-MYC) with the immunoglobulin heavy chain (IgH), which places C-MYC under the control of the of IgH locus[175]. The translocations that are found in lymphoid malignancies are caused by problems that occur during the programmed DSB repair in V(D)J combination. These translocations result when paired DSBs on separate chromosomes are fused together erroneously[174].

It is known that the MRN complex has roles in proper V(D)J recombination. While homozygous mutations for the *Mre11*, *Rad50*, or *Nbs1* genes cause embryonic lethality in mice, humans and mice with hypomorphic mutations in *MRE11* or *NBS1* present clinically with immunodeficiency, likely caused by defects in DSB repair pathways due to loss of the MRN complex[100, 135, 162, 176]. Furthermore, mouse thymocytes with a hypomorphic mutation in *Mre11* have an increased number of trans-rearrangements, suggesting that mutations in MRN can lead to aberrant V(D)J recombination[164]. Finally, studies have shown there is an increase of unrepaired coding-ends associated with hypomorphic *Mre11* or *Nbs1* mutations in developing mouse lymphocytes[177]. Taken together, understanding the mechanisms of how malignant translocations occur and the DNA repair mechanisms by which they are prevented are important for the

development of therapeutics in lymphoid cancers. The work in Chapter IV elucidates roles for the MRN complex, and specifically MRE11 nuclease activity, in lymphogenesis.

The KU70/KU80 heterodimer

The KU70/80 heterodimer is an abundant, highly conserved DNA-binding protein and sensor of DNA DSBs[178]. KU70/80 is found in both prokaryotes and eukaryotes and plays essential roles in the maintenance of genome integrity. In eukaryotes, KU is a heterodimer consisting of two subunits, KU70 and KU80[179, 180]. KU is best known for its central role as the initial DNA end-binding factor in classical NHEJ pathway. KU binds DSBs with high affinity through its central ring structure formed by the intertwined strands of the KU70 and KU80 subunits[179, 180]. It is not currently known how KU is ejected from DSBs. At a DSB, KU directly and indirectly interacts with several NHEJ factors and processing enzymes[26]. For example, KU binds and activates the DNA-PKcs kinase and, together, KU and DNA-PKcs make up the DNA-PK holoenzyme[179]. KU is central to the protection of organisms through its participation in NHEJ to repair DSBs generated through V(D)J recombination[113]. KU also functions to prevent tumorigenesis and senescence since *Ku*-deficient mice show increased cancer incidence and early onset aging[113, 181]. It is unclear why eukaryotes have two DNA DSB sensors (MRN and KU). The work in Chapter II contributes to a better understanding of the interplay between these two sensor complexes.

Cell cycle regulation

Cyclins and CDKs control cell cycle progression

The cell cycle is controlled by several mechanisms to ensure correct cell division. Cell division consists of two consecutive processes: DNA replication and segregation of replicated chromosomes into two separate cells. Cell division is divided into several stages[182]. Mitosis is the process of nuclear division and interphase is the stage between two M phases. The stages of mitosis include prophase, metaphase, anaphase, and telophase. Interphase includes the G1, S, and G2 phases. Replication of DNA occurs during S phase. S phase is preceded by a gap called G1 during which the cells prepares for DNA synthesis and is followed by G_2 during which the cell prepares for mitosis. Cells in G1 can enter a resting state called G0 before committing to DNA replication. Cells in G0 account for a major portion of non-growing, non-proliferating cells in the human body[182].

The transition from one cell cycle phase to another is regulated by different proteins[183]. One of the key sets of regulatory proteins is the CDKs which are a family of serine/threonine protein kinases. When activated, CDKs induce downstream processes by phosphorylating targeting proteins. CDK protein levels remain stable during the cell cycle, in contrast to their activating proteins, the cyclins. Cyclin protein levels rise and fall during the cell cycle; binding of cyclins activate their CDK partners. Different cyclins are required at different phases of the cell cycle. The three D cyclins (Cyclin D1, Cyclin D2, and Cyclin D3) bind to CDK4 and to CDK6; CDK-Cyclin D complexes are essential for entry into

G1. Cyclin E associates with CDK2 to regulate progression from G1 into S phase. Cyclin A also binds with CDK2 and this complex regulates progression in the S phase. Furthermore, Cyclin A complexes with CDK1 to promote entry into M phase. Mitosis is also regulated by Cyclin B binding to CDK1[183].

In addition to cyclin binding, CDK activity is also regulated by phosphorylation on conserved threonine and tyrosine residues. CDK activity can be counteracted by cell cycle inhibitory proteins, called CDK inhibitors, which bind to CDKs alone or to CDK-Cyclin complexes. The intracellular localization of different cell cycle-regulating proteins also contributes to cell cycle progression[183] (Figure 1.6).

Regulation of CDK activity

Regulation hierarchies are critical in ensuring the completion of one cell cycle phase before the beginning of the next. The mechanisms that control these important tasks are highly conserved in evolution. Kinase activity of all CDKs requires the binding of a positive regulatory subunit known as the cyclins[184]. Both cyclins and CDKs are subject to post-translational regulation by phosphorylation. The assembly of a CDK with its corresponding cyclin yields only a partially active complex and full activity occurs after phosphorylation of the CDK on a conserved threonine residue close to the ATP-binding cleft (Threonine 172 in CDK4/6, Threonine 160 in CDK2, and Threonine 161 in CDK1)[183]. The activating threonine residue is located in a loop of amino acids called the T-loop that blocks access of ATP to the catalytic domain. Analysis of the crystal structure

of CDK2-Cyclin A complexes indicates that the CDK-Cyclin interaction causes a conformational change in the CDK, making the T-loop more accessible for the activating phosphorylation[185]. The phosphorylation causes a further conformational change in the T-loop, making the catalytic cleft fully accessible to ATP[185]. In addition to greatly stimulating kinase activity, the activating threonine phosphorylation has also been suggested to enhance stability of some CDK-Cyclin complexes[185].

CAK or CDK-activating kinases are responsible for catalyzing the activating threonine phosphorylation. The phosphorylation of CDKs by CAK is antagonized by the action of a specific phosphatase known as KAP. Phosphorylation of CDKs can also negatively regulate their kinase activity[183]. The inhibitory phosphorylations occur near the N termini of all CDKs, specifically on Tyrosine 15 of CDK2 and CDK1, and on Tyrosine 17 on CDK4 and CDK6. In the case of CDK2 and CDK1, there is also a second inhibitory phosphorylation involving Threonine 14[186]. Phosphorylation of these sites results in inhibition of CDK activity even in the presence of the CAK-catalyzed activating phosphorylation. Wee1-like protein kinase (WEE1) and membrane-associated tyrosine-and-threonine-specific cdc2-inhibitory kinase (MYT1) have been identified as the kinases responsible for the phosphorylation of the inhibitory sites on CDK2 and CDK1[187-189]. WEE1 and MYT1 are bifunctional kinases that can phosphorylate both tyrosine and threonine residues, although WEE1 shows a preference for Tyrosine 15 and MYT1 for Threonine 14[187-189]. The inhibitory phosphorylations of CDKs are removed by the action of the CDC25 family of protein phosphatases[190]. Interestingly, the

CDC25 proteins are themselves substrates of CDKs-Cyclins, and their phosphorylation stimulates the phosphatase activity. For example, CDK2-Cyclin E phosphorylates M-phase inducer phosphatase 1 (CDC25A) at the G1/S transition, and the activity of CDC25A is necessary for CDK2-Cyclin E activation, thus creating a positive feedback loop. The activity of CDC25A is high from the G1/S boundary to mitosis, and is required for the activation of CDK2-Cyclin A. The CDC25 phosphatases also play important roles in the DNA damage-induced checkpoints[191-193].

Another important mechanism for regulating CDK-Cyclin activity is their interaction with inhibitory proteins. Several CDK inhibitors have been identified and can be divided into two families: the INK4 family (p16, p15, p18, and p19) and the CIP/KIP family (p21, p27, and p57)[194-196]. The INK4 family of CDK inhibitors block the progression of the cell cycle in G1 by binding either CDK4 or CDK6 and inhibiting the action of Cyclin D[194, 195]. The CIP/KIP family of CDK inhibitors act more broadly and can block the activity of all CDK-Cyclin complexes and are characterized for their role as negative regulators of G1 phase cell cycle progression[197]. The expression of p21 is under the transcriptional control of the p53 tumor suppressor gene[198]. p21 is activated by p53 in response to DNA damage and is also upregulated during replicative cellular senescence[199]. Proliferating cell nuclear antigen (PCNA) has been found in p21/CDK/Cyclin complexes, suggesting a function in DNA replication or DNA repair[197, 199]. The expression and activation of p27 increases in response to transforming growth factor β (TGF-β), contributing to growth arrest[200, 201]. p27

appears to regulate CDK-Cyclin complexes in a manner similar to that of p21[202].

Elimination of both positively and negatively acting cell cycle effectors is important for orderly cell cycle progression. The ubiquitin-mediated proteasome system is the main pathway employed for the degradation of cell cycle factors[203]. Two structurally and functionally similar complexes, the Skp-Cullin-Fox (SCF) complex and the anaphase-promoting complex (APC), target specific cell cycle components for ubiquitination at discrete points in the cell cycle[203]. The SCF complex is employed at the end of G1, through S and into early G2 phase, whereas APC becomes active at the end of G2 and mediates the transition through mitosis[204, 205]. Both SCF and APC act as E3 ubiquitin ligases leading to degradation of marked complexes (Figure 1.7).

CDK substrates

When CDKs are active, target proteins become phosphorylated on CDK consensus sites, resulting in changes that are physiologically relevant for cell cycle progression. The most frequently studied target is the substrate of CDK4/6-Cyclin D, the retinoblastoma tumor suppressor gene (RB)[206]. During G1, RB becomes phosphorylated and this leads to disruption of the complex with the histone deacetylase protein (HDAC) and release of the transcription factors E2F-1 and DP-1, which positively regulate the transcription of genes whose products are required for S-phase progression, including Cyclin A, Cyclin E, and CDC25[207, 208]. During G1/S the CDK2-Cyclin E complex phosphorylates its inhibitor p27,

inducing its proteasome-dependent degradation[209, 210]. CDK2-Cyclin E phosphorylates Histone H1 and this activity may be important for chromosome condensation required during DNA replication[211, 212]. Histone H1 is also a substrate for CDK1-Cyclin B[213]. Cyclin A-dependent kinases regulate initiation of DNA replication by phosphorylation of DNA polymerase α primase[214]. Other CDK substrates include CDKs own regulators WEE1 and CDC25, and cytoskeletal proteins such as nuclear lamins and microtubules, which are required for correct mitosis[188, 190, 215]. Interestingly, CDK2 has also been shown to have a role in suppressing MYC-induced senescence, which suggests that inhibition of CDK2 may have therapeutic potential in MYC-driven cancers[216].

CDK2 and the DNA Damage Response

To further understand the functions of CDK2 *in vivo*, researchers generated *Cdk2* knockout mice. Surprisingly, these mice were viable, indicating CDK2 is not an essential gene in the mouse[217]. Alternatively, knockout of *Cyclin A2* in mice leads to early embryonic lethality[218]. It was also found that CDK2 is required for germ cell development, as both male and female *Cdk2*-null mice are sterile. CDK2-null MEFs proliferate but enter in a delayed fashion into S phase. Ectopic expression of CDK2 in CDK2-null MEFs rescued the delayed entry into S phase[217]. Thus, loss of CDK2 affects the timing of S phase, suggesting that CDK2 is involved in regulating progression through the mitotic cell cycle. It is not known why CDK2 is dispensable for cell proliferation. Perhaps phosphorylation of CDK2 substrates is not essential or there are other kinases that

can phosphorylate essential CDK2 substrates. Likewise, other CDKs may step in and substitute in the case of another non-functional CDK.

Only recently has CDK2 catalytic activity been associated with DNA damage response pathways. Two studies identified NBS1 as a CDK substrate[170, 219]. The authors found that NBS1 is phosphorylated by CDK2 on Serine 432 in human whole-cell extracts. Mutant forms of NBS1 could not be modified by CDK2 and were defective in protecting cells from death due to IR-induced DNA damage. These studies determined that CDK2 activity has a role in the DNA damage response and that NBS1 is a specific target of CDK2 within the DNA repair machinery[170, 219]. More questions remain such as what other DNA repair proteins are targets of CDK phosphorylation and what aspects of the MRN-complex function depend on CDK activity.

The repair of DNA DSBs is tightly regulated by the cell cycle. In G1, the error-prone NHEJ is the predominant repair pathway, while in S and G2, there is an increase in repair of DSBs by HR which is mostly error-free[220]. However, there are still gaps in our knowledge about how DSBs are directed for repair by these two different and competing repair pathways. In mammals, CTIP is a key protein responsible for cell cycle regulation of resection during HR and is regulated by cell-cycle specific post-translational modifications[37, 221, 222]. During G1, CTIP protein levels are suppressed by proteasome-mediated degradation and as cells enter S phase, the protein levels rise again[223]. Additionally, CTIP is a substrate of S phase specific CDK activity and contains two well-conserved CDK phosphorylation sites[35]. Researchers found that CTIP is required for repair of

DSBs by HR in S and G2 and that the function of CTIP in HR is dependent on CDK-dependent CTIP phosphorylation on Serine 327 and subsequent interaction with BRCA1[224-226]. Interestingly, cells expressing CTIP protein that cannot be phosphorylated at Serine 327 are defective in HR. This data creates a model by which phosphorylation of CTIP on Serine 327 as cells enter S phase/recruitment of BRCA1 functions as a molecular switch to shift DSB repair from NHEJ to the more efficient repair pathway, HR[225]. Additionally, CTIP is phosphorylated on Threonine 847; this phosphorylation event is required for CTIP to localize to DSBs and modification of this site leads to increased genomic instability[35]. A recent study identified novel (lesser-conserved) CDK phosphorylation sites on CTIP and phosphorylation of these sites induce association of CTIP with NBS1[227]. They went on to show that CTIP is phosphorylated by ATM after DNA damage. Notably, CDK-mediated phosphorylation of CTIP is required for CTIP to be phosphorylated by ATM after DNA damage, which is important for end resection and HR by promoting recruitment of BLM and EXO1 to DSBs[227].

It is known that MRE11, of the MRN complex, provides nuclease activities required for initiation of resection during HR[163]. Interaction of MRN with both CTIP and BRCA1 assist in these functions[226]. CTIP interaction is required to enhance MRE11 nuclease activity, and for efficient resection *in vivo*, CTIP must interact with BRCA1. Thus the formation and dissolution of the MRN-CtIP-BRCA1 complex during S and G2 regulates resection capacity[35]. Our lab further investigated the relationship between CTIP and the MRN complex. Buis et al. discovered that the C-terminus of MRE11 (which is absent in an inherited patient

allele causing ATLD) controls the phosphorylation status and overall levels of CTIP protein, and through this process, controls formation of the MRN-CtIP-BRCA1 complex during S and G2[140]. Furthermore, the C-terminus of MRE11 directly interacts with CDK2 bound to Cyclin A. This data supports the hypothesis that the MRE11-CDK2 interaction facilitates the phosphorylation of CTIP in normally dividing cells[140]. It will be important in future studies to understand how the interaction between CDK2 and the MRE11 C-terminus might activate CDK2 substrates other than CTIP. My work in Chapter III elucidates functions of this interaction during the DNA damage response.

Finally, CDK2-Cyclin A has been found to have roles in regulating V(D)J recombination[228]. The generation of lymphocyte-specific antigen receptors by V(D)J recombination is controlled by levels of RAG2 protein, which accumulates in G1 phase of the cell cycle, but is rapidly degraded at the G1-S transition, continuing until the next entry to G1[229]. This ensures that the DSBs generated by RAG2 can be repaired correctly by NHEJ, which is active during G1, and thus prevents genomic instability. A recent report showed that CDK2-Cyclin A opposes RAG2 accumulation (conversely, RAG2 is induced by p27 and other CDK inhibitors)[228]. More specifically, they found that Threonine 490 on RAG2 is an essential CDK phosphorylation site, and its mutation abolishes degradation of RAG2 protein[228]. In thymocytes of mice expressing a mutation in RAG2 Threonine 490, aberrant recombination events accumulate. These observations suggest that restriction of RAG2 accumulation to the G0 and G1 cell cycle phases promote correct repair of V(D)J recombination intermediates by NHEJ[228]. The

cell cycle dependence of V(D)J recombination suggests it may be interesting in future studies to determine how genetic disruption of the MRE11-CDK2 interaction alters lymphogenesis and V(D)J recombination in mice.

The cell cycle checkpoints

Overview of cell cycle checkpoints

DNA damage checkpoints are biochemical pathways that delay or arrest cell cycle progression in response to DNA damage[92]. Originally, a checkpoint was defined as a specific point in the cell cycle when the integrity of DNA was examined or checked before allowing progression through the cell cycle. More recently, checkpoint responses have become more clearly defined as constant surveillance and response systems that continuously monitor the integrity of the genome and control cell-cycle progression[92]. DNA damage checkpoints are positioned before the cell enters S phase (G1-S checkpoint), during S phase (intra-S-phase checkpoint), and after DNA replication (G2-M checkpoint). The DNA damage checkpoints have three components: sensors, signal transducers, and effectors[230, 231]. Although the G1/S, intra-S, and the G2/M checkpoints are distinct, the damage sensor molecules that activate the various checkpoints appear to either be shared by all three pathways or to play a primary sensor role in one pathway and a backup in the others[69, 231, 232] (Figure 1.8). My thesis is focused on understanding the role of the MRN complex in the intra-s-phase checkpoint, described in the next few sections.

DNA replication in S-phase

DNA replication or DNA synthesis is the process of producing two identical copies of DNA from one original DNA molecule and occurs in the S phase of the cell cycle[233]. The process of replication is highly conserved across evolution. Accurate replication of the genome ensures the transmission of genetic material from parental cells to daughter cells and is critical for the survival all organisms. DNA is made up of a double helix structure consisting of two complementary strands, which are separated during the replication process[234]. Each strand serves as a template for the production of its counterpart, a process which is known as semi-conservative replication[233].

DNA replication begins at specific locations in the cell called origins of replication[235]. First, assembly of the pre-replication complex occurs during late M phase and early G1 when CDK activity is low, thus ensuring that DNA replication occurs only once per cell cycle[235]. In eukaryotes, the origin recognition complex (ORC), which includes cell division cycle 6 (CDC6) and DNA replication factor CDT1 (CDT1), recognize the origins of replication and load the mini-chromosome maintenance (MCM2-7) helicase onto the origin, forming the pre-replication complex[236-238]. MCM2-7 is phosphorylated by cell division cycle 7-relted protein kinase (CDC7) and various CDKs, which displaces CDC6 and recruits MCM10[237]. Next, the factors DNA replication complex GINS protein PSF1 (GINS) and cell division control protein 45 homolog (CDC45) are recruited to the origin[236]. CDC45 then recruits key components of the replisome, including the

replicative DNA polymerase α and its primase[239, 240]. At this point, DNA replication can now begin.

First, the two strands of the DNA double helix are separated by the MCM helicase. Unwinding of the DNA at the origin and synthesis of new strands results in the formation of replication forks, which form bi-directionally from the origin[241]. Once the two strands are separated, primase adds RNA primers to the template strands[242]. DNA polymerase synthesizes the new strands in a 5' to 3' direction by adding nucleotides that complement each template strand[240]. The leading strand is continuously extended by the DNA polymerase with high pocessivity, while the lagging strand is extended discontinuously from each primer, which forms Okazaki fragments[243]. Next, RNase removes the primer RNA fragments. DNA Ligase then fills in a single nick on the leading strand and several nicks on the lagging strand[244]. This completes the formation of a newly replicated DNA molecule.

The intra-S-phase checkpoint

The intra-s-phase checkpoint is activated to delay progression through S-phase allowing time for DNA repair to occur and to prevent genomic instability[245]. There are in fact several types of intra-s-phase checkpoints. The first is activated by DNA DSBs in genomic loci outside of active replicons. Thus, the DSB-induced intra-s-phase checkpoint acts independently of replication forks. The other intra-s-phase checkpoints include the replication-dependent intra-s-phase checkpoint (or replication checkpoint), and the S-M checkpoint, which both respond to problems with replication[245]. To clarify, my thesis research is

focused on the DSB-induced intra-s-phase checkpoint (herein called “the intra-s-phase checkpoint”). Previous studies reported two parallel branches of the intra-s-phase checkpoint that slow down ongoing DNA synthesis in S-phase[191, 192]. The first is controlled by the ATM signaling machinery, which controls CHK2-mediated degradation of CDC25A (Figure 1.9A-B). Inhibition of CDK2 activity downstream of this pathway blocks loading of CDC45 onto chromatin. CDC45 is a protein required for the recruitment of DNA polymerase α into the assembled pre-replicons, so the inhibition of CDK2 activity prevents the initiation of new origin firing[191, 192]. Another branch of the intra-s-phase checkpoint involves ATM-mediated phosphorylation of NBS1 and SMC1[246]. Mutations of ATM phosphorylation sites on NBS1 (Serine 343) and SMC1 (Serines 957 and 966) result in S-phase checkpoint defects[246]. This data highlights that understanding the functional relationships between ATM and the MRN complex can provide further insight into the mechanisms that regulate abnormal ionizing radiation responses. Importantly, it is possible that there are several mechanisms or pathways that induce the s-phase checkpoint. My thesis was aimed at elucidating how the MRN complex, through interaction with CDK2, functions in S-phase checkpoint control and suggests a new model for induction of an S-phase checkpoint through functions of the MRE11 C-terminus.

Radioresistant DNA Synthesis

Defects in the intra-s-phase checkpoint result in the inability of cells to reduce their rate of replication after IR[247]. This phenomenon, known as

radioresistant DNA synthesis, was first reported in cells derived from patients with ataxia-telangiectasia who have mutations in the *ATM* gene[248]. The RDS phenotype has also been reported in cells with defects in other components of the checkpoint, including ATM, ATR, CHK2, CHK1, CDC25A, NBS1, MRE11, SMC1, MDC1, 53BP1, and BRCA1 and BRCA2[64, 191, 192, 249-254]. The assay for measuring RDS has become a “gold standard” to test whether or not any new candidate protein is required for the intra-s-phase checkpoint[247]. In this assay, the rate of replication is measured by incorporation of radioactively labeled thymidine, followed by a quantification method such as liquid scintillation or autoradiography[230, 232]. Currently, DNA synthesis rate measurements have remained an attractive first-line diagnostic tool for patients with A-T.

DNA repair and the cell cycle as targets in cancer therapy

Cancer is a disease of genomic instability and uncontrolled cell division[1, 2]. Thus, targeting DNA repair mechanisms and the cell cycle components are likely promising avenues for cancer therapy (Figure 1.10). Deregulation of both CDKs/Cyclins and DNA repair genes occur frequently in certain types of cancer. As examples, Cyclin E1 or E2 amplifications are key oncogenic events in uterine and ovarian cancers[255, 256]. The CDK inhibitor p27 is downregulated in many cancers, although the genetic loss of p27 is fairly rare[257, 258]. Xeroderma pigmentosum and trichothiodystrophy are autosomal recessive disorders caused by defects in nucleotide excision repair[259]. The disease Fanconi Anemia (FA), which leads to bone marrow failure, is caused by defects in the FA proteins which

are responsible for repairing DNA intra- and inter-strand crosslinks[260]. Finally, both BRCA1 and BRCA2, which are involved in the HR repair pathway, are found in approximately 5-7% of hereditary breast cancers[261].

Translating all this knowledge into successful clinical development of CDK and DNA repair inhibitors has been challenging. The first generation of CDK inhibitors developed were relatively nonspecific and are often referred to as pan-CDK inhibitors (i.e. Roscovitine developed by Cyclacel)[262]. A second-generation class of CDK inhibitors was next developed with the aim of increasing selectivity for CDK1 and CDK2 and increasing potency. Many seemed to be promising in preclinical trials, but only a few progressed past Phase 1 clinical trials[202].

In recent years, the focus has transitioned to targeting CDK4 and CDK6, specifically. The actions of CDK4/6 through phosphorylation of RB control the transition from G1 to S phase[206]. The rationale for targeting CDKs 4 and 6 is that these inhibitors would elicit G0/G1 arrest, act directly on RB to suppress gene expression and proliferation, and would be actionable in tumors that exhibit deregulation of CDK4 and CDK6 activity as opposed to other CDKs[263]. Three CDK4/6 inhibitors, abemaciclib, palbociclib, and ribociclib, have reached early phase clinical trials. Results from Phase III trials investigating palbociclib in patients with advanced-stage estrogen receptor (ER)-positive breast cancer have demonstrated substantial improvement in progression-free survival[264].

Radiation therapy continues to be a main treatment in a variety of cancers. Exposure of tumors to ionizing radiation causes DSBs, which are deleterious to

cell survival. Chemotherapy is also a popular treatment which is used to stop or slow the growth of cancer cells. However, chemotherapy is a systemic therapy that affects the entire body. Thus, new treatments are needed to avoid the harsh side effects of both radiation and chemotherapy. Perhaps the most promising DNA repair therapy is that of poly(ADP-ribose) polymerases (PARP) inhibitors, which are used in a therapeutic strategy termed “synthetic lethality”[265]. PARPs are a family of nuclear proteins that function in DNA damage recognition. PARP1 has been shown to either directly or indirectly influence the repair of DNA in multiple pathways, including base-excision repair (BER), single-stranded break (SSB) repair, and alternative-NHEJ[266]. The PARP1 inhibitor known as Olaparib (AstraZeneca) suppresses SSB and BER repair pathways, thus causing PARP to be trapped onto SSBs, resulting in replication fork-stalling, fork collapse, and DSBs[267, 268]. In normal cells, these breaks would be repaired by the mostly error-free HR pathway. However, in tumor cells mutant for either BRCA1 or BRCA2, which are crucial for repair by HR, the breaks are instead repaired by error-prone pathways, causing catastrophic damage and cell death[266-268]. Olaparib is currently FDA approved for treatment in recurrent ovarian cancer and germline-BRCA, HER2-negative metastatic breast cancer[269].

Challenges remain, including development of highly selective molecules against other CDKs and DNA repair factors, understanding mechanisms of drug-resistance to these inhibitors, determining appropriate patient populations, and gaining a better understanding of both the pharmacology and biology that will assist in development of useful drug combinations.

Thesis Summary

The specific aims of my thesis were to further uncover how MRN functions in DNA damage kinase activation, cell cycle checkpoint control, and tumorigenesis. In Chapter II, I set out to gain a deeper understanding of the interplay between the two main DNA damage kinase sensors, MRN and the KU heterodimer, along with their respective kinases, ATM and DNA-PKcs. We hypothesized that loss of both DNA DSB sensors would prevent DNA damage response signaling. To test our hypothesis, we utilized mouse models in our lab that are deficient for MRN, KU, or both. To our surprise, we found that loss of both MRN and KU still allowed for ATM signaling, implying that MRN is not absolutely required for activation of ATM. In Chapter III, I worked to uncover the biological functions of the interaction between the DNA repair protein MRE11 and the cell cycle kinase CDK2. We hypothesized that the MRE11-CDK2 interaction is a component of the intra-s-phase checkpoint. To test our hypothesis, we utilized mutants genetically lacking the MRE11-CDK2 interaction in our experiments. Interestingly, we found that the MRE11 C-terminus is important for maintaining normal levels of CDK2 activity. In Chapter IV, we aimed to uncover roles for the MRN complex in oncogenesis. We hypothesized that mice lacking the MRN complex or MRE11 nuclease activity in B cells would development B-cell specific tumors. To test our hypothesis, we generated mice lacking MRE11 or MRE11 nuclease activity in B-lymphocytes and monitored them for tumor development. We found that, contrary to our hypothesis, the mice did not develop pro-B lymphomas, implying that MRN is not a classic

tumor suppressor, and that certain functions of the complex, such as the nuclease activity of MRE11, may be required for progression to cancer.

Figure 1.1. The Hallmarks of Cancer. Depiction of the characteristics necessary for tumor development. This thesis is focused on investigating the roles of genome instability/mutation and evading growth suppressors in cancer. Adapted from Hanahan and Weinberg, Cell, 2011.

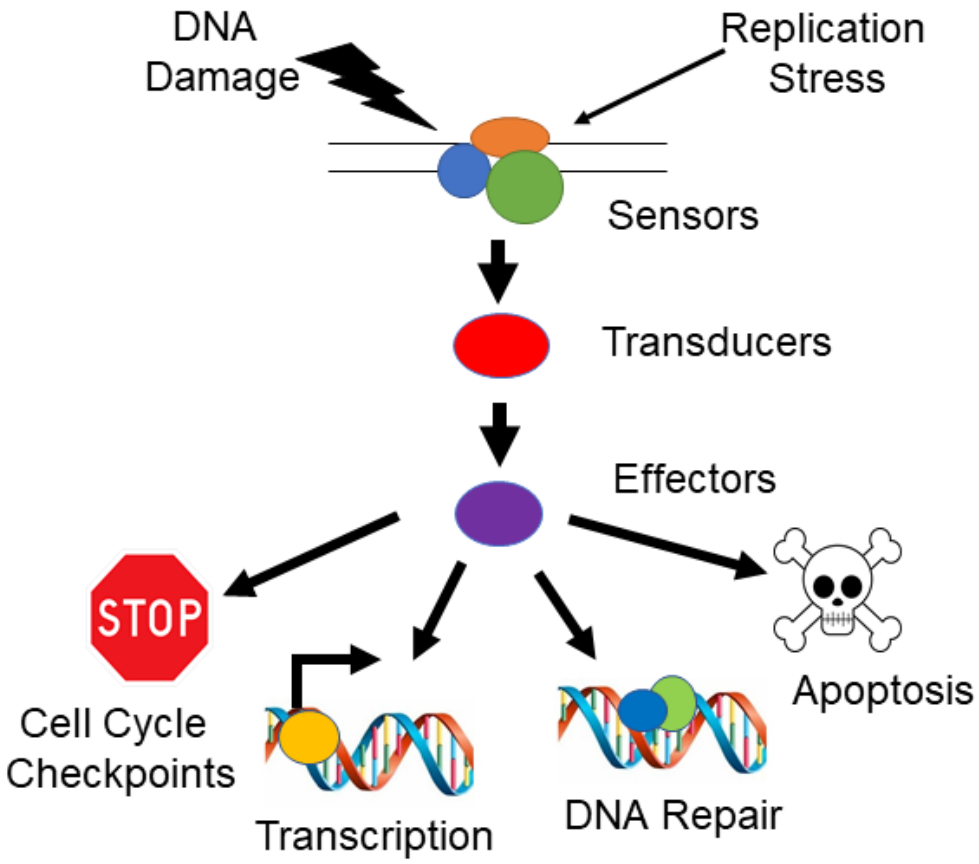
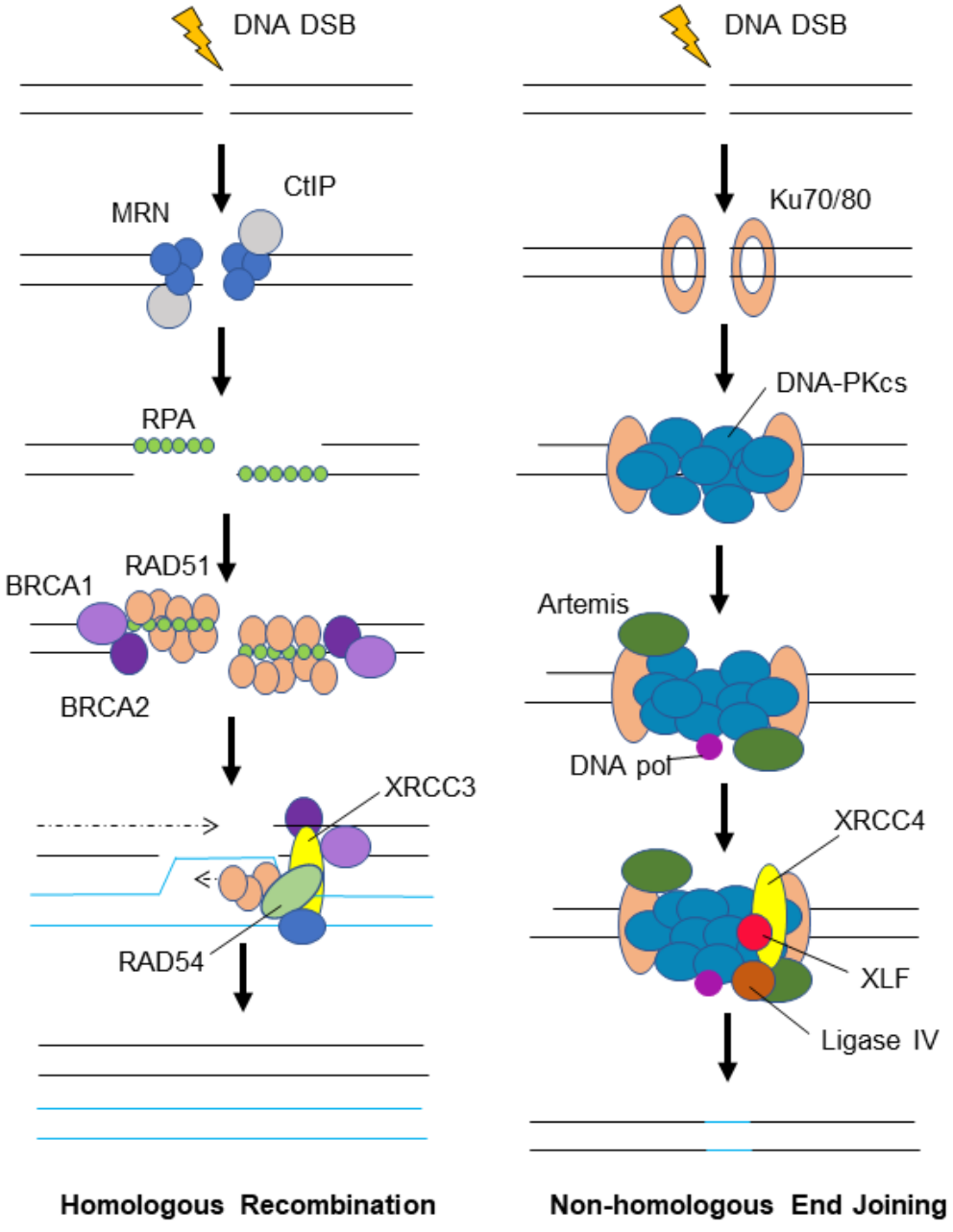


Figure 1.2. A model for the DNA damage response signaling pathway. Diagram showing the signaling pathways involved in responses to DSBs. Sensor proteins such as MRN or KU sense and bind the DSB. Transducer proteins are recruited and activated, such as the ATM kinase. ATM phosphorylates effector proteins, which are often recruited to the sites of damage, and initiate a large array of cellular processes, including: cell cycle checkpoints, DNA repair, transcriptional activation, and if the damage cannot be fixed, the cell will undergo programmed cell death or apoptosis. The DNA damage response is an important barrier to cancer development. Adapted from Zhou et al., Nature, 2000.



Homologous Recombination **Non-homologous End Joining**

Figure 1.3. The DNA double-strand break repair pathways: HR and NHEJ. During HR, the MRN complex recognizes the DSB and recruits and activates the ATM kinase. MRE11, along with CTIP, resect the ends to yield a 3' ssDNA overhang. The ends are further processed by the activities of EXO1 and BLM helicase. The ssDNA is then rapidly bound by RPA, which is then replaced by RAD51 to form a nucleofilament that is important for homology searching and strand invasion. Canonical NHEJ involves recruitment of the KU70/80 heterodimer to the DSB and activation of DNA-PKcs. Ligation of broken DNA ends is mediated by DNA ligase IV and XRCC4. Adapted from Brochier et al., Journal of the American Society for Experimental NeuroTherapeutics, 2013.

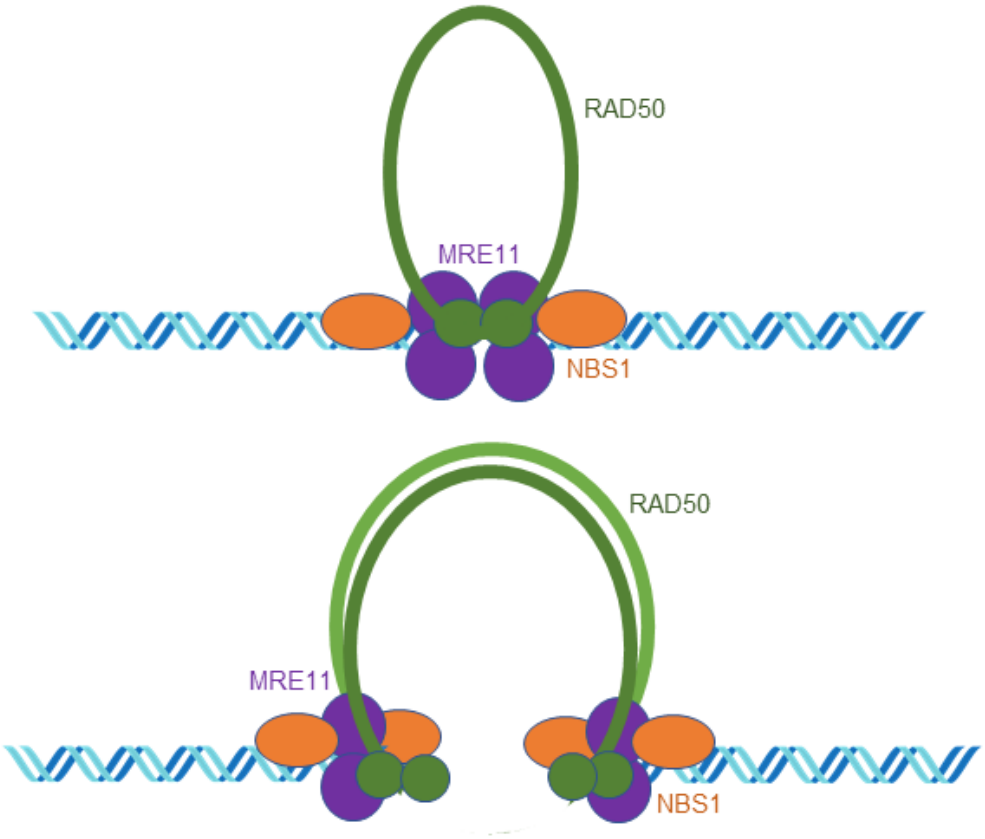


Figure 1.4. The MRE11/RAD50/NBS1 Complex. The MRN complex is a multi-protein complex consisting of three proteins: MRE11, RAD50, and NBS1. MRE11 and RAD50 form a heterotetramer that bridges DNA ends together over both long and short distances. Adapted from Lavin et al., Biomolecules, 2015.

Disease	Gene	Cancer Pre-Disposition	Immuno-deficiency	Other	Mouse Knockout
Ataxia Telangiectasia	ATM	Yes	Yes	Neuronal degeneration Ataxia	Viable
Ataxia Telangiectasia Like Disorder (ATLD)	MRE11	?	?	Neuronal degeneration	Lethal
Nijmegen Breakage Syndrome (NBS)	NBS1	Yes	Yes	Mental retardation Microcephaly	Lethal
Nijmegen Breakage Like Disorder	RAD50	?	No	Mental retardation Microcephaly	Lethal

Figure 1.5. Inherited deficiencies in DNA DSB signaling. There are a number of diseases caused by deficiencies in DNA repair factors. Ataxia-telangiectasia (A-T) is caused by mutations in the *ATM* gene and is clinically characterized by cancer predisposition, immunodeficiency, ataxia, and neuronal degeneration. *Atm*-null mice are viable. Mutations in the *MRE11* gene cause the disease ataxia-telangiectasia-like disorder (ATLD). Patients with ATLD have neuronal degeneration resulting in ataxia, but do not have telangiectasia or immunodeficiency. A connection between ATLD and cancer predisposition still remains unclear. Mutations in the *NBS1* gene cause Nijmegen breakage syndrome which is characterized by immunodeficiency, cancer predisposition, mental retardation, and microcephaly. Nijmegen breakage like-disorder is caused by mutations in the *RAD50* gene, which causes mental retardation and microcephaly in patients. Knockout of any genes in the MRN complex leads to embryonic lethality in mice, so human patients with mutations in the MRN complex represent a form of hypomorphism, in which functions of the MRN complex necessary for development remain intact.

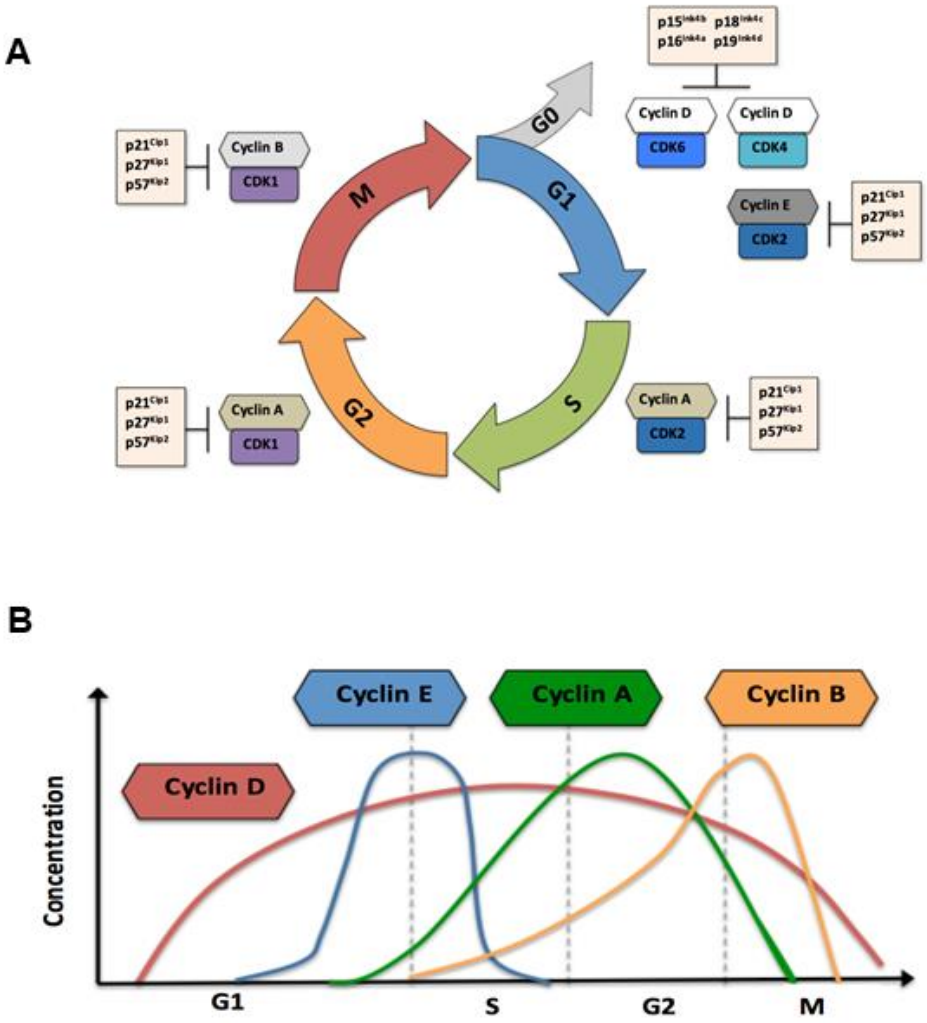


Figure 1.6. Cyclins and CDKs control the cell cycle. (A) Depiction of the cell cycle. Early G1 is controlled by CDK4 and CDK6 binding to Cyclin D. Entry into S phase is controlled by CDK2 binding to Cyclin E. Progression in the S phase is regulated by CDK2 binding to Cyclin A. G2 is controlled by CDK1 binding to Cyclin A. CDK1 bound to Cyclin A controls mitosis. Also shown are the CDK inhibitor proteins that bind to each CDK/Cyclin complex in order to inhibit its activity. **(B)** Diagram of the cyclin levels in each phase of the cell cycle. Cyclin D is low in G1 and M phase and high in S and G2. Cyclin E levels are high in G1 and early S. Cyclin A is high in S and early G2. Cyclin B levels rise M phase. Adapted from Hochegger et al., Nature Reviews Molecular Cellular Biology, 2008.

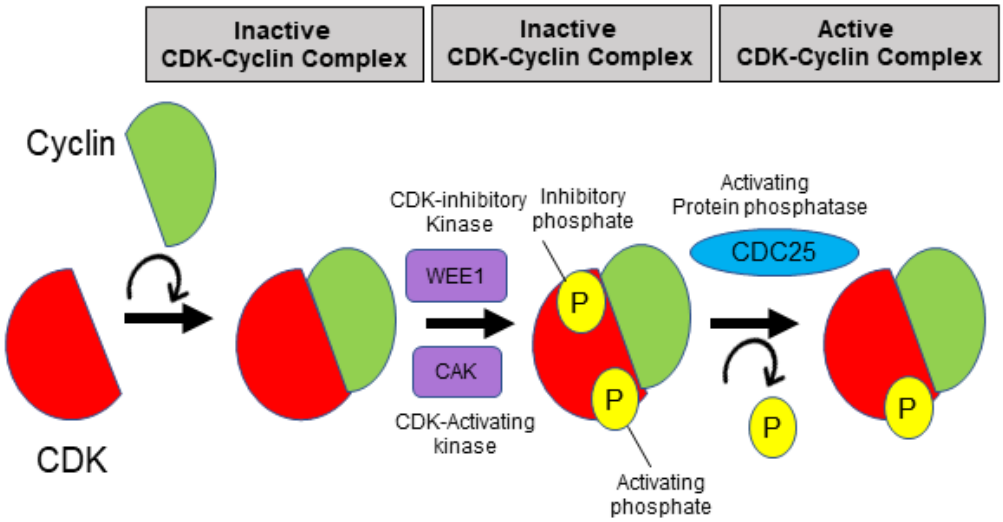


Figure 1.7. Regulation of CDK activity. The activity of CDKs are regulated in a variety of ways. CDKs become fully active when they are bound by their cyclin partners and are phosphorylated on an activating phosphate group by CAK kinases. CDKs become inactive when not bound by their cyclin partners and upon phosphorylation on inhibitory phosphate groups. WEE1 is an example of a kinase that phosphorylates inhibitory phosphates to deactivate CDKs. CDC25 phosphatases de-phosphorylate the inhibitory phosphorylation to stimulate CDK activity. Adapted from Molecular Biology of the Cell, 4th Edition, 2008.

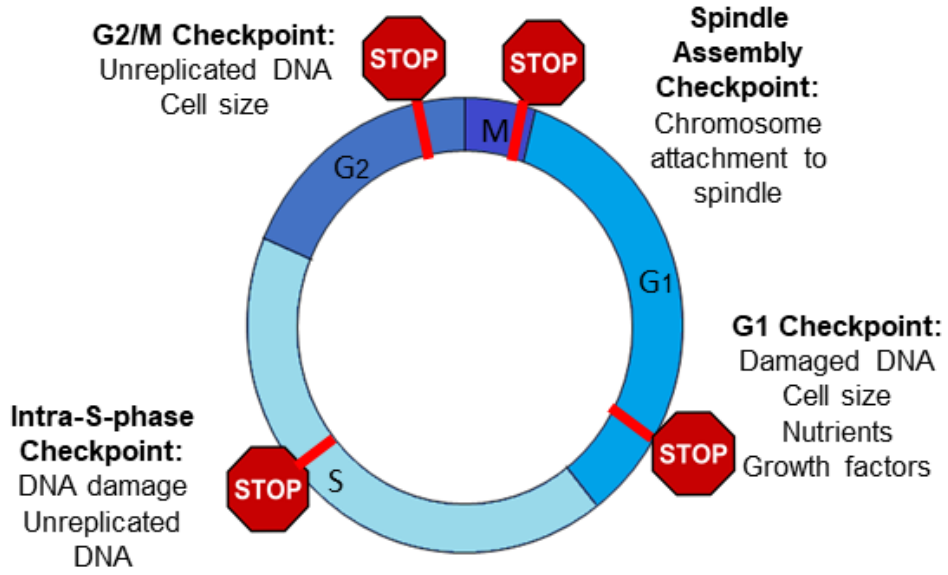


Figure 1.8. The cell cycle checkpoints. The cell cycle checkpoints are points in the eukaryotic cell cycle at which progression of the cell into the next stage of the cell cycle is halted until conditions are more favorable. The G1 checkpoint monitors for DNA damage, cell size, nutrients, and growth factors before entering S-phase at which DNA is replicated. The intra-S-phase checkpoint is activated to delay progression through S-phase allowing time for DNA repair to occur before mitosis. The G2/M checkpoint checks for unreplicated or damaged DNA. Finally, the spindle assembly checkpoint monitors for chromosome misalignment. All cell cycle checkpoints are important for preventing genomic instability. Adapted from Filho et al., Frontiers in Cellular and Infection Microbiology, 2017.

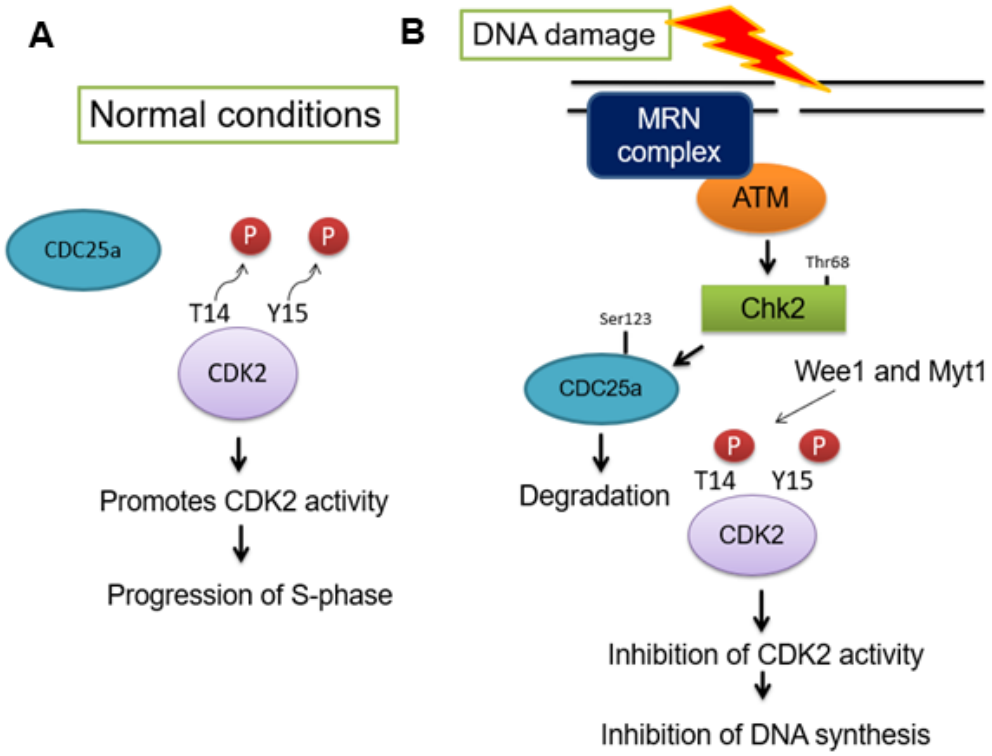


Figure 1.9. The ATM-dependent S-phase checkpoint pathway. Several S-phase checkpoint pathways have been previously described. Here is a diagram of the players currently known to be involved in the ATM-dependent intra-S-phase checkpoint pathway as described by Falck et al., in 2000. In general, the S-phase checkpoint detects and responds to DNA damage and replication stress in S-phase, allowing time for DNA repair to occur and to prevent genomic instability. **(A)** Under normal conditions, the phosphatase CDC25A de-phosphorylates inhibitory phosphorylation on CDK2, promoting CDK2 activity and progression of S-phase. **(B)** Upon DNA damage, the MRN complex senses the DSB and facilitates recruitment and activation of the ATM kinase. ATM-dependent phosphorylation of CHK2 on Threonine 68 leads to phosphorylation of the phosphatase CDC25A on Serine 123, marking it for its degradation. WEE1 and MYT1 phosphorylate CDK2 on inhibitory phosphorylation sites, which inhibits CDK2 activity, thus stalling progression in S-phase. Adapted from Falck et al., Nature, 2000.

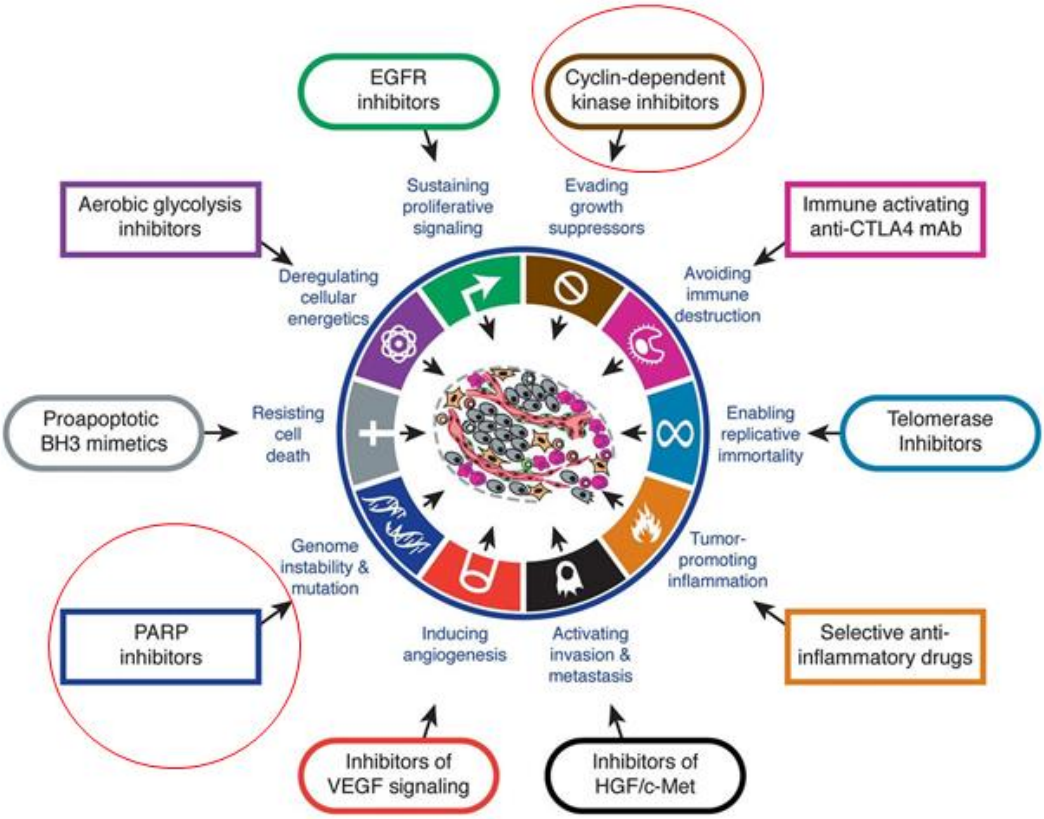


Figure 1.10. Therapeutically targeting the Hallmarks of Cancer. Drugs targeting each of the acquired factors necessary for tumor growth and progression are being developed and tested in clinical trials. In some cases, these drugs are already being used in the clinical setting for the treatment of cancer. For example, PARP inhibitors have shown success in treating breast cancer patients with mutations in the BRCA genes. Adapted from Hanahan and Weinberg, Cell, 2011.

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Chapter II

Recruitment and Activation of the ATM Kinase in the Absence of DNA-Damage Sensors*

Abstract

DNA double-strand breaks (DSBs) are among the most toxic forms of DNA damage, and if left unrepaired, are potent causes of genome instability. The DNA damage response is a complex network of signaling events that initiates cellular responses important for the repair of DNA damage. There are two main kinases that control rapid responses to DNA double-strand breaks: ataxia-telangiectasia mutated kinase (ATM) and DNA-dependent protein kinase, catalytic subunit (DNA-PKcs). Previous knowledge in the field has shown that ATM is recruited and activated by the MRE11-RAD50-NBS1 (MRN) sensor complex at DNA DSBs. Likewise, DNA-PKcs is recruited and activated by the sensor KU70/KU80 heterodimer complex at DSBs. Although much is known about how MRN and KU

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MJM contributed to Figures 2.2-2.6
AJH contributed to Figures 2.1-2.2, and 2.6

along with their respective kinases function in isolation, it is unclear how they might influence each other at DSBs during the DNA damage response.

To gain further insight into the interplay between the two main DNA damage sensor complexes, our lab generated mouse cells containing targeted mutant alleles of *Mre11* and/or *Ku70* and used pharmacologic kinase inhibition of ATM and DNA-PKcs on the cells in *in vitro* experiments. Interestingly, we found that ATM can be activated by DSBs in the absence of MRN. When MRN is deficient, DNA-PKcs efficiently substitutes for ATM in facilitating local chromatin responses. In the absence of both MRN and KU, ATM is still recruited to chromatin where it phosphorylates H2AX and triggers the G2-M cell-cycle checkpoint, although the DNA-repair functions of MRN are not restored. These results suggest that, in contrast to straightforward recruitment and activation by MRN and KU, the interchange between the two DNA damage sensors and their respective kinases is more complicated than previously thought. We also show that MRN is not absolutely required for recruitment and activation of ATM.

Introduction

DNA DSBs are highly toxic forms of DNA damage. In eukaryotes, there are two major DNA DSB repair pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ). Before DNA repair can occur, sensor proteins must detect the damage and initiate the DNA damage response (DDR), a complex network of signaling events that lead to initiation of cellular processes including DNA repair, cell cycle checkpoints, and in some cases, apoptosis or senescence. The failure to repair DNA DSBs can be detrimental to cells and organisms alike, leading to carcinogenesis as well as DNA repair disorders with an array of clinical manifestations such as sensitivity to radiation, suppression of the immune system, a predisposition to cancer, and neurodegeneration[1].

At the apex of the DNA damage response are the two major, highly conserved DNA-damage sensor complexes, MRN and KU[2, 3]. MRN and KU are both thought to recruit and activate kinases that are members of the phosphatidylinositol 3-kinase-related kinase (PIKK) family of serine/threonine kinases: ATM and DNA-PKcs, respectively[4, 5]. Although there is much known about how both MRN and KU function on their own to recruit and activate ATM and DNA-PKcs, there is still a great deal to learn about how they may interact with each other during the DNA damage response (Figure 2.1A). Furthermore, it is unclear why in most metazoans two separate kinases are needed in the DNA damage response and how activation of either kinase is accomplished in the context of apparently redundant sensors.

The ATM kinase functions at the top of the DSB signaling cascade and has a vast number of substrates that coordinate many cellular outcomes. Inherited mutations in *ATM* cause the disease ataxia-telangiectasia (A-T), patients of which present with a predisposition to cancer, immunodeficiency, and neurological degeneration[6]. The previous understanding of ATM activation was that MRN is absolutely required for DSB sensing and binding, and recruitment and activation of ATM[7-9]. Roles for MRN in the activation of ATM are supported by studies showing: 1) mutations in *MRN* result in human diseases with A-T like features, 2) there is defective activation of ATM observed in MRN-mutant and deficient cells, and 3) there is enhanced ATM kinase activity observed in the presence of MRN *in vitro[6, 8, 10-12]*.

The KU70/KU80 heterodimer complex also binds DSBs, and activates the DNA-PKcs kinase[13]. Together, KU and DNA-PKcs make up the DNA-PK holoenzyme[13]. KU is a very abundant cellular protein and exhibits high affinity for DNA DSBs[14]. Unlike ATM and its many functions, the primary role of DNA-PK is direct participation in DSB repair in the NHEJ pathway[15]. Mice deficient in *DNA-PKcs*, and those lacking *Ku70* or *Ku80*, are immune-deficient and have defective V(D)J recombination, which requires NHEJ[16, 17].

The cellular responses to DSBs are sophisticated and involve many proteins that must be tightly regulated to generate the necessary outcome. A deeper understanding of these processes is important for uncovering the pathogenic mechanisms of cancer and for the designing of successful cancer treatments. However, answering these questions will be a difficult challenge. To

gain mechanistic insight into the earliest events in the mammalian DSB response, we examined cells lacking one or both DSB-sensing complexes and treated them with inhibitors for one or both PIKK DSB kinases. We found cells with normal levels of MRN are dependent on ATM for facilitating chromatin responses after ionizing radiation-induced damage. In cells that lack MRN, DNA-PKcs efficiently substitutes for ATM. Most intriguingly, we discovered that removal of KU in the absence of the MRN complex restores several ATM-dependent responses, demonstrating that MRN is not absolutely required for recruitment and activation of ATM.

Results

Generation of MRN-deficient and MRN/KU-deficient cells

We disabled the MRN complex through use of mouse embryonic fibroblasts (MEFs) that originally contained one *Mre11*-null allele and one conditional allele. Exposure to Cre recombinase via adenovirus converts these cells to *Mre11*$^{-/-}$, which also causes deficiency of RAD50 and NBS1 (termed MRN deficiency) most likely because of instability of the proteins in the absence of MRE11 (Figure2.1B)[10]. Control cells used for experiments were initially *Mre11*$^{Cond/+,}$ which we converted to *Mre11*$^{-/+}$ via Cre recombinase in parallel with other genotypes to control for effects of addition of Cre adenovirus. Through mouse breeding, Andrea Hartlerode, a senior investigator in the lab, also generated MEFs of these MRN genotypes combined with germline knockout of *Ku70* (*Ku70*$^{-/-}$). Removal of *Ku70* does not require use of a conditional allele, and it causes deficiency of its heterodimeric partner *Ku80* (termed *Ku-deficiency*), as previously described (Figure 2.1C)[17].

Early recruitment of MRN complex and KU to DNA damage

To better understand the multifaceted interplay between the DNA damage sensor complexes in the immediate aftermath of DSB induction, we utilized live-cell imaging coupled with microirradiation to examine the localization of both MRN and KU to sites of DNA damage. Andrea Hartlerode and I performed these experiments together. We first transfected GFP-tagged human NBS1 into MEF

cells to track MRN recruitment to laser-induced damage in real-time. MRN was recruited within 15 to 30 seconds after damage, and KU deficiency did not appear to affect its recruitment (Figure 2.2A). To track KU, we transfected GFP-tagged KU70 and KU80 and found that KU recruitment was faster than laser stripes could be generated. Instead of taking time-lapse images, we pulsed the laser at a single location while simultaneously recording video. We detected KU at damage sites within 1 second after damage, and it continued to accumulate for approximately 10 seconds in most cells. Loss of the MRN complex did not appear to affect KU recruitment (Figure 2.2B). Therefore, immediately after DNA damage, the absence of either sensor does not substantially affect overall accumulation of the other.

Localization of KU to DSBs in the absence of the MRN complex

To gain a deeper understanding of KU localization to sites of DSBs in the absence of MRE11 at later timepoints following DSB induction, I examined KU foci formation in both control and MRE11-deficient cells on a time-course of 5 minutes to 2 hours after ionizing radiation treatment (Figure 2.3A). We hypothesized that KU might localize at DSBs longer or faster, or otherwise may be more abundant in MRE11-deficient cells versus control cells. Even though KU is an abundant protein and a major DSB sensor in mammalian cells, it has been nearly impossible, in the past, to visualize it at single DSB sites in cells by fluorescence microscopy. Stephen Jackson's group showed in 2013 that this inability to detect KU and other DNA repair proteins at DNA ends arises because a large portion of these proteins

are associated with chromatin via RNA. Thus, the group developed a new technique which combines RNAse A and detergent-based pre-extraction with high-resolution microscopy, allowing for detection of KU at DSBs in cells[18]. In my hands, KU70 foci were too small to count, and so I utilized ImageJ to quantify the fluorescent intensity of nuclei as a measure of KU70 foci formation at DSBs (Figure 2.3B).

My results showed minimal KU70 foci formation in the absence of DNA damage in both control and MRE11-deficient cells. Cells lacking the MRN complex displayed a higher baseline of KU70 fluorescent intensity, which could be reflective of an increased number of spontaneous DSBs when MRN is absent. When DNA damage is induced via ionizing radiation, KU70 is rapidly recruited to DSBs (within 5 minutes) in both control and MRE11-deficient cells. Interestingly, both control and *Mre11*$^{-/-}$ cells reached the same maximal peak of KU70 foci formation at 5 minutes post-ionizing radiation. KU70 foci formation began to decrease at 30 minutes and plateaued out to two hours following ionizing radiation treatment in both cells lines. Interestingly, KU70 mean fluorescent intensity returned to its baseline after 5 minutes post-ionizing radiation in *Mre11*$^{-/-}$ cells, but remained increased relative to control cells. I examined KU70 foci formation in *Ku70*$^{-/-}$ cells as control for antibody specificity, and as expected, these cells formed no KU70 foci.

My results did not support our hypothesis that in the absence of the MRN complex, KU70 is recruited faster or more abundantly to DSBs, which would suggest an antagonistic model of interplay between the two DSB sensor proteins.

Possible explanations may be that I missed the maximal peak of KU70 foci formation in *Mre11*$^{-/-}$ cells or spontaneous DSBs mask the true baseline of KU70 foci formation in MRE11-deficient cells. Overall, my data show that the absence of MRE11 does not appear to affect accumulation of KU70 foci formation at DSBs after DNA damage induction.

Phosphorylation of H2AX in the absence of DNA damage sensors

To further understand ATM recruitment in the absence of DSB sensors, I investigated one of the earliest events in the DSB response, which is phosphorylation of the histone variant H2AX (also known as γH2AX)[19, 20]. Phosphorylation of H2AX occurs upon DNA DSB formation, as well as in response to replication stress, and is dependent on ATM. Depending on cell type, H2AX is phosphorylated for megabase regions surrounding each side of a DSB and serves as a platform for more extensive chromatin changes, such as recruitment of DNA repair proteins to the chromatin, that are required to spread DNA-damage responses[21, 22]. Phosphorylated H2AX forms nuclear foci at sites of DNA DSBs, which can be visualized as punctate dots, using immunofluorescent microscopy. Therefore, I set out to observe γH2AX foci formation in MRE11 and KU mutant MEFs, treated with irradiation to induce DSBs and with different combinations of PIKK kinase inhibitors.

In each genotype, most cells (65-80%) contained ten or more γH2AX foci, and the morphologies of these foci were all similar (Figure2.4A). In control cells,

the ATM inhibitor markedly decreased γH2AX foci formation, whereas DNA-PKcs inhibition had little effect on foci formation. In contrast, I saw the opposite pattern in MRN-deficient cells, where the DNA-PKcs inhibitor reduced γH2AX foci formation to near-background levels. These results are consistent with models in the literature depicting a requirement for MRN in ATM recruitment/activation under normal conditions, and it also demonstrates that DNA-PKcs can efficiently substitute for ATM in phosphorylating H2AX when MRN is absent[8, 9]. Surprisingly, in *Mre11*$^{-/-}$*Ku70*$^{-/-}$ cells, I observed substantial levels of γH2AX after ionizing radiation treatment and this phosphorylation event returned to being ATM-dependent. Thus, ATM is activated in the absence of both MRN and KU and is recruited to chromatin in the vicinity of DSBs to phosphorylate H2AX. This data is consistent with western blot analysis done by Andrea Hartlerode comparing levels of phosphorylated H2AX (data not shown). Quantitation of γH2AX immunofluorescent foci 30 minutes before and after ionizing radiation treatment was graphed from 3 independent experiments (Figure 2.4B).

53BP1 localization to DSBs in the absence of DNA damage sensors

To determine whether this sensor-independent ATM activity is restricted to H2AX phosphorylation, we examined other DNA damage response events. Additional ATM-dependent events such as phosphorylation of KAP1 and SMC1 were also restored in *Mre11*$^{-/-}$*Ku70*$^{-/-}$ cells (data not shown). As with H2AX phosphorylation, the degree of phosphorylation was less than in control cells, but was fully ATM-dependent. H2AX phosphorylation can also lead to recruitment of

p53-binding protein (53BP1), which, in turn, is important for recruiting other repair factors. 53BP1 is a key regulator of DSB repair pathway choice[23]. During G1 it promotes NHEJ mediated repair by antagonizing long-range DNA end resection which is essential for HR. Interestingly, 53BP1 can still form foci in *H2ax*$^{-/-}$ cells and it has several pathways of recruitment to DSBs, all of which are not entirely dependent on ATM[23]. Thus, I examined the formation of 53BP1 foci in MRE11 and KU70 mutant cells along with pharmacologic kinase inhibition after ionizing radiation treatment (Figure 2.5A). Not surprisingly, I found that only a portion of 53BP1 foci formation was dependent on the DNA damage kinases. The graph shows quantitation of 53BP1 foci 30 minutes before and after ionizing radiation treatment from 3 independent experiments (Figure 2.5B).

Rapid MDC1 recruitment in the absence of MRN and KU

After H2AX is phosphorylated, it is bound by the protein called mediator of DNA damage checkpoint 1 (MDC1)[24, 25]. MDC1 then promotes recruitment of DNA damage response and checkpoint proteins to the chromatin surrounding DSBs, ultimately leading to initiation of the G2-M checkpoint[26]. To gain further insight into the newly discovered MRN-independent ATM activation, Andrea Hartlerode and I examined GFP-tagged MDC1 recruitment with live-cell imaging after laser microirradiation[27]. This approach allows for visualization of recruitment within seconds to minutes after damage, which is much faster than timescales permitted with immunofluorescence-based experiments. Thus, ATM

activation can be compared within the earliest timeframe of the DNA damage response.

In control cells, we found that MDC1 recruitment occurred by 15 seconds and this recruitment was dependent on ATM[24]. When MRN was deficient, MDC1 accumulated as rapidly and intensely as control cells, but this recruitment switched to being dependent on DNA-PKcs. Thus, even during the rapid events that occur immediately after DNA damage, DNA-PKcs can locally substitute for ATM when MRN is not there. *Mre11*$^{-/-}$*Ku*$^{-/-}$ cells exhibited recruitment of MDC1 similar to control cells, and this was, strikingly, ATM-dependent (Figure 2.6). Therefore, even within the first minute of the DSB response, ATM can function in the absence of both DNA damage sensor complexes.

Discussion

We have shown in this study that when MRN is absent, removal of KU restores several ATM functions, including its ability to phosphorylate H2AX. Other functions restored include ATM's ability to relocate to chromatin and to activate the G2-M checkpoint (data not shown). However, DNA-repair functions of MRN were not restored in the absence of KU (data not shown). These findings indicate that MRN is not absolutely required for recruitment and activation of ATM, but perhaps instead it functions to maximize or sustain ATM-dependent DSB responses. Unlike a simple recruitment model previously believed in the field, activation of ATM appears to be strongly influenced by the complex interplay between MRN and KU occurring within seconds after DNA damage.

Studies in the past have shown that KU and MRN can compete for binding to DSBs *in vitro* and that KU must be removed from DNA ends to allow for HR to occur[28-30]. Together, these results suggest some level of competition between MRN and KU, however, our live-cell imaging studies suggest otherwise. We show that both sensors bind DSBs rapidly, with KU binding first and MRN joining shortly after. Neither complex appears to require the other for recruitment to DSBs. Importantly, the absence of one sensor does not measurably affect the recruitment of the other, even within the earliest time-points after DNA damage induction. These observations lead us to speculate that there is a level of opposition in the functions of MRN and Ku at DNA ends that does not just involve simple competition for initial binding.

We also uncovered several instances of evidence that support the notion that MRN, although it is not absolutely essential for initial ATM activation, maximizes and sustains ATM-dependent responses. Phosphorylation of ATM substrates KAP1 and SMC1 in MRN- and KU-deficient cells was not as robust as in control cells (data not shown). Live-cell imaging of γH2AX-dependent MDC1 recruitment demonstrated that MDC1 was rapidly recruited to sites of laser-induced damage in cells of all the genotypes examined, but this recruitment was slightly decreased in MRN- and KU-deficient cells 15 seconds after damage. This level of recruitment is near the limit of detection of the instrument used for this study, and thus interpretations must be considered with care. Overall, western blot analysis of ATM substrates that measure responses over longer times suggested a more substantial delay than seen in the live-cell imaging, but still point to the conclusion that MRN works to maximize/sustain ATM functions.

For MRN to perform its ATM-dependent functions, it must be capable of overcoming the more rapid binding of KU, which protects the ends of DNA[28-30]. The two KU subunits form a basket-shaped structure that threads onto DNA ends. Once bound, KU can slide down the DNA strand, allowing for more KU molecules to thread onto the end. One hypothesis is that MRN could eject KU from the DSB by converting DSBs into ssDNA ends, which are a poor substrate for KU binding[29, 31]. The MRN complex possesses two activities that can generate ssDNA: the nuclease activities of MRE11 and ATP-dependent unwinding of DNA by RAD50[32]. While our studies point to the latter possibility over the first, future studies will need to investigate both questions (Figure 2.7).

The newly uncovered MRN-independent activation of ATM presented in this study represents a notable advance in our understanding of the early DSB response. The ability of ATM to function in the absence of MRN helps to explain studies in mice that lack the critical ATM-interacting domain of *Nbs1*, which display astonishingly minimal AT-like phenotypes[33, 34]. The strong requirement for MRN only in the presence of KU suggests that a key function for MRN in ATM activation is to oppose KU at DSBs to expose DNA ends. This idea is supported by previous *in vitro* studies, including one from *Xenopus* egg extracts that showed that ATM can be activated in the absence of MRN, but only when relatively higher levels of DNA ends were added[35-37]. Further studies will be needed to determine whether another unknown protein is required for recruitment and activation of ATM or to see if ATM is simply activated by the presence of DNA ends *in vivo*.

The disease known as ataxia-telangiectasia-like disorder (ATLD) is caused by mutations in *MRE11* and is phenotypically fairly similar to the disease ataxia-telangiectasia (A-T) caused by mutations in *ATM*[38, 39]. ATLD is a rare disease characterized clinically by progressive cerebellar degeneration resulting in ataxia. Patient cells have defects in cell cycle checkpoints, increased sensitivity to radiation, and an in increase in chromosomal aberrations, all consistent with a defect in DNA repair[38-40]. Interestingly, our study proposes a novel way to treat patients with MRN deficiency. Hypothetically, it could be possible to restore ATM activation in individuals with inherited *MRN*-deficiencies by pharmacologically

inhibiting KU function. More studies will have to be conducted to test this intriguing hypothesis.

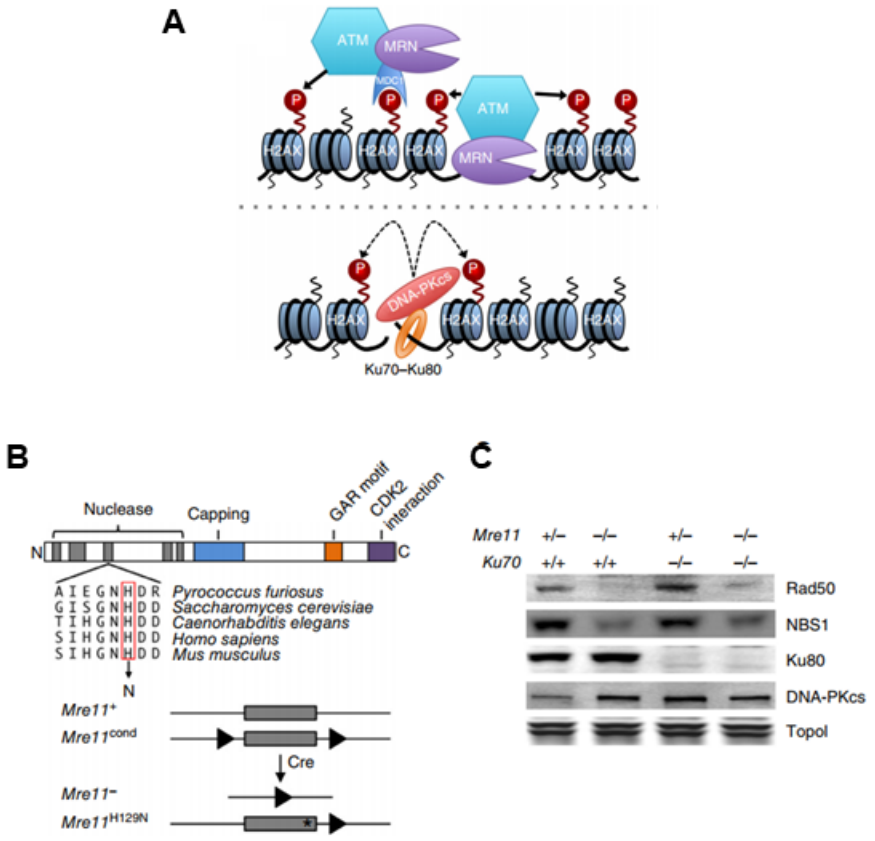

Figure 2.1. The early response to DNA DSBs. **(A)** Prevailing model of the DNA DSB response in which MRN senses DSBs and activates ATM kinase leading to phosphorylation of the histone variant H2AX; and the KU70/KU80 heterodimer senses DSBs and activates DNA-PKcs kinase also leading to phosphorylation of H2AX. **(B)** Schematic of the mammalian MRE11 domain structure and our lab's engineered mouse *Mre11* germline alleles. **(C)** Confirmation immunoblot of MRE11-deficient, KU70-deficient, and combined MRE11/KU70-deficient cell lines. Topoisomerase I (TopoI) is the protein loading control.

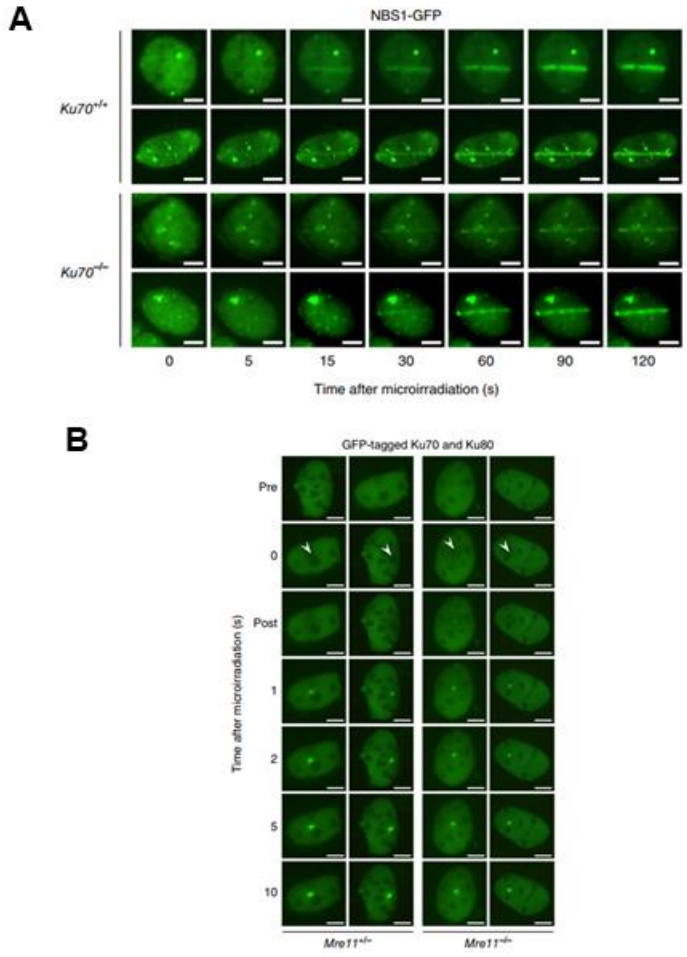

Figure 2.2. MRN and KU relocate to DSBs independently in the early DSB response. Live-cell imaging of MRN **(A)** and KU **(B)** relocation to laser microirradiation-induced damage. Two representative cells are shown per genotype, from a total of 45 and 88 cells per genotype in A and B, respectively; three independent experiments were performed. Scale bars, 10 μM. **(A)** GFP-tagged NBS1, monitored in live cells by time-lapse photography during florescence microscopy. **(B)** GFP-tagged KU70 and KU80, monitored in live cells via florescence microscopy video capture. Individual frames corresponding to indicated points (left) are shown. Arrowheads indicate spot of laser target.

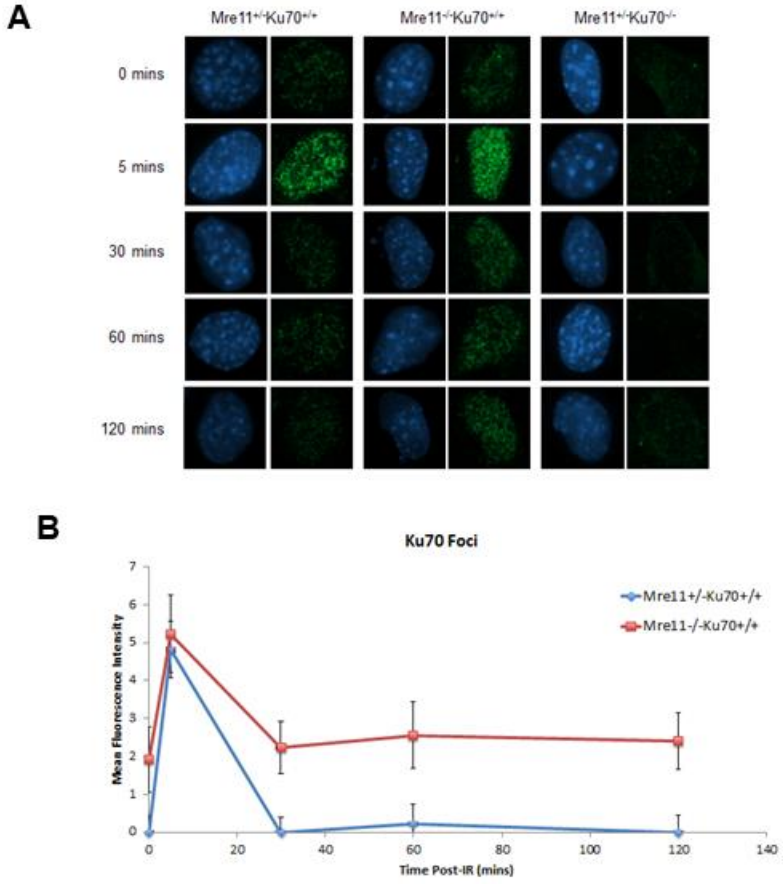

Figure 2.3. Localization of KU to DSBs in the absence of the MRN complex in response to DSBs. (A) KU70 foci formation in control (MRE11-wildtype), MRE11-deficient, and KU70-deficient cells after 0, 5, 30, 60, and 120 minutes post-ionizing radiation. Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Genotypes are as shown. **(B)** Mean Fluorescence Intensity of KU70 nuclear foci formation in control and MRE11-deficient cells after 0, 5, 30, 60, and 120 minutes post-ionizing radiation. Error bars represent standard error of the mean across three independent experiments.

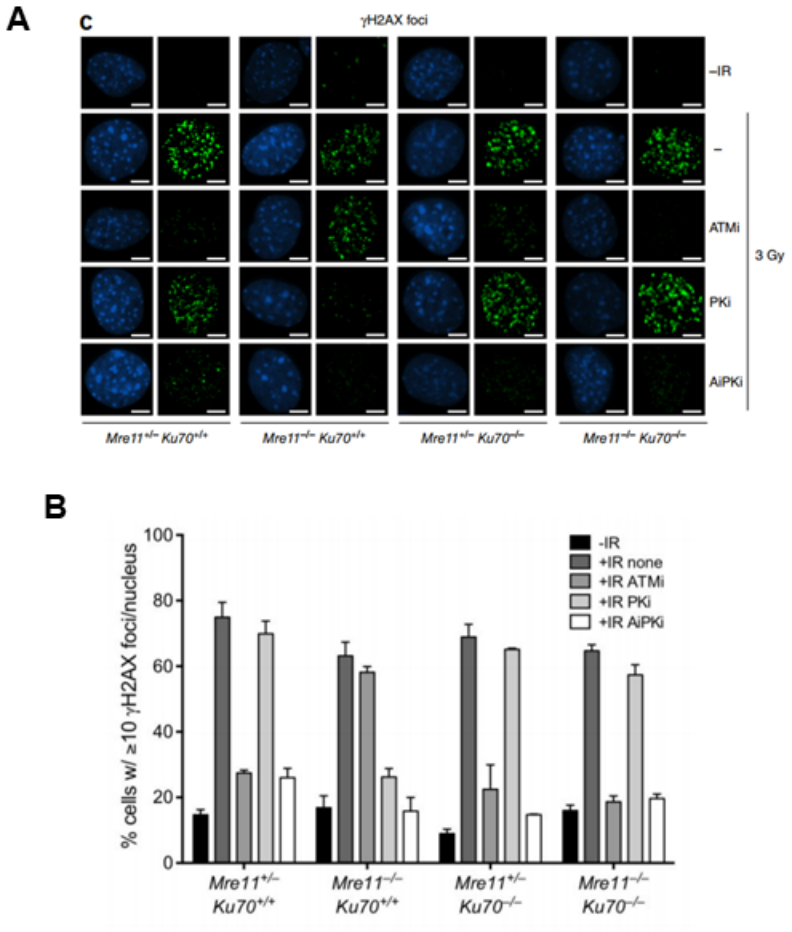

Figure 2.4. Phosphorylation of H2AX in the absence of DNA damage sensors. (A) γH2AX immunofluorescent foci (green) 30 minutes after 3Gy ionizing radiation treatment, with or without pre-treatment with kinase inhibitors (ATMi, ATM inhibitor; PKi, DNA-PK inhibitor; and AiPKi, both ATM and DNA-PK inhibitors). Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Genotypes are as shown. Scale bars, 10μM. **(B)** Graph shows the quantification of γH2AX immunofluorescent foci 30 minutes after ionizing radiation. Data shown are means and standard error the mean of 3 independent experiments. The majority of cells (65% to 80%) contained 10 or more γH2AX foci and the morphology of these foci were similar amongst all genotypes.

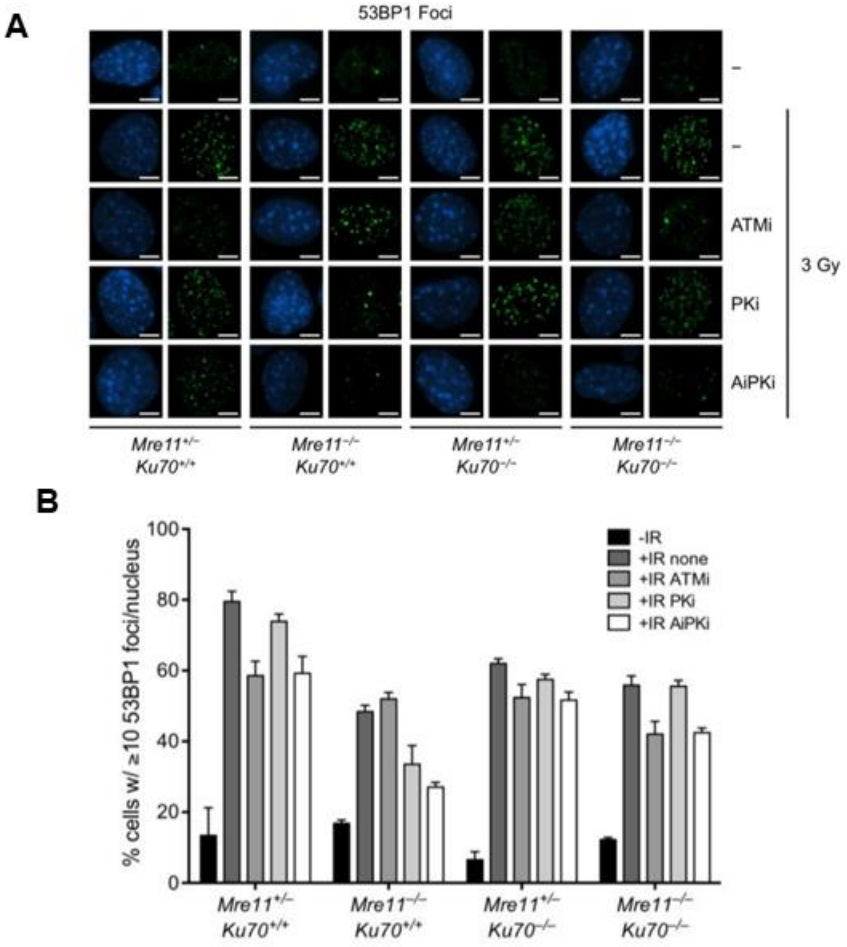

Figure 2.5. 53BP1 foci formation in the absence of MRN and KU. (A) 53BP1 immunofluorescent foci (green) 30 minutes after 3Gy ionizing radiation treatment, with or without pretreatment with kinase inhibitors (ATMi, PKi, and AiPKi). Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Genotypes are as shown. Scale bars, 10 μM. **(B)** Quantification of 53BP1 foci. Data shown are means and standard error of the mean of 3 independent experiments. Note that only a portion of 53BP1 foci formation is dependent on DNA damage kinases.

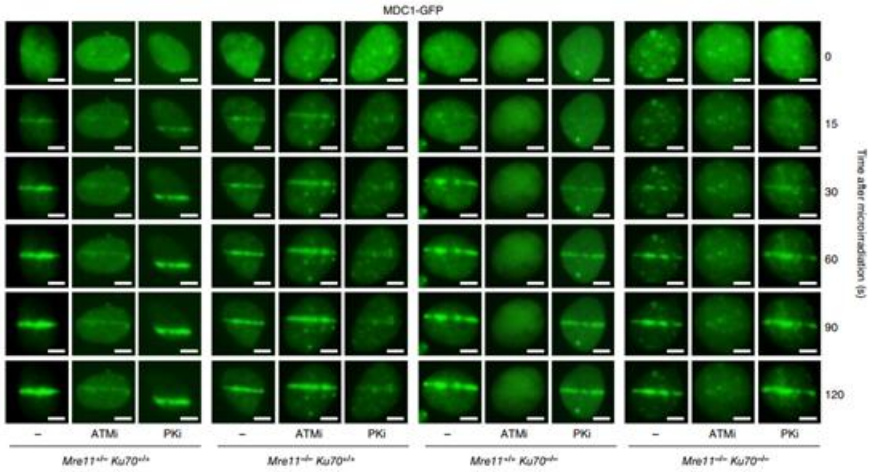

Figure 2.6. Recruitment of MDC1 in the absence of both MRN and KU. Here we show recruitment kinetics of the MDC1 protein. The BRCT domain of MDC1 was tagged with GFP and expressed in cells of the indicated genotypes. Live cells with or without pre-treatment with kinase inhibitors (ATMi and DNA-PKi) were exposed to laser micro-irradiation and monitored by time-lapse photography at the indicated times. Panels show one representative cell out of a total of 30-40 cells per genotype and condition; 3 independent experiments were performed. Scale bars, 10 μM.

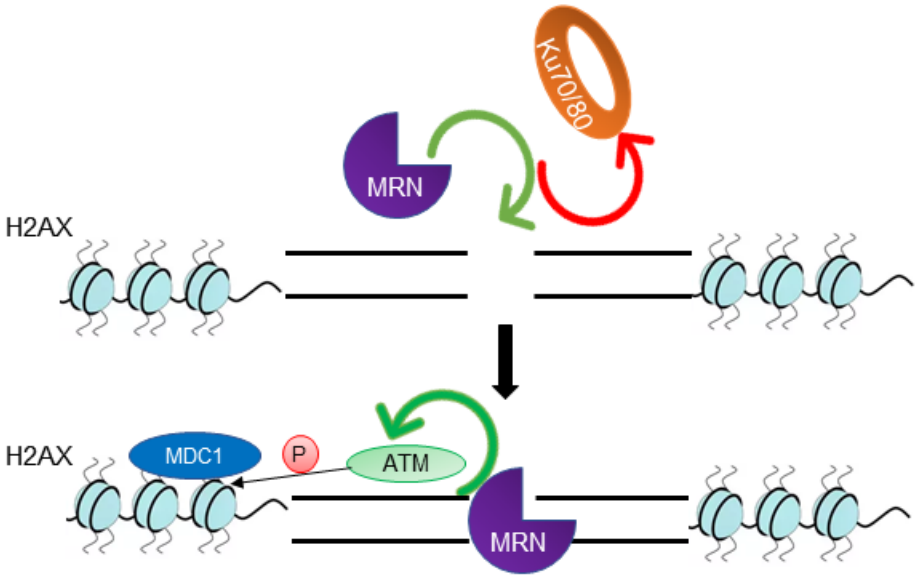

Figure 2.7. Model for opposition of MRN and the KU heterodimer at double-strand breaks. In control cells, we demonstrate that phosphorylation of H2AX and recruitment of MDC1 to DSBs is dependent on ATM. In the absence of MRN, these events are DNA-PKcs-dependent. Strikingly, in the absence of both DSB sensor complexes, phosphorylation of H2AX and recruitment of MDC1 still occur and are ATM-dependent. Additionally, we show that KU is recruited to DSBs before MRN. Together, this data suggests there is some form of opposition between the two main DSB sensors to bind DSBs first. In this model, MRN must somehow oppose KU to bind the DSB, which leads to activation of ATM, phosphorylation of H2AX, and recruitment of MDC1. Importantly, our model implies that direct interaction between MRN and ATM is not required for activation of ATM, but that MRN works to maximize ATM activation.

Materials and Methods

Cell culture

The following MEF genotypes were used in this study: *Mre11*$^{+/-}$*Ku70*$^{-/-}$, *Mre11*$^{-/-}$*Ku70*$^{+/+}$, *Mre11*$^{+/+}$*Ku70*$^{-/-}$, and *Mre11*$^{-/-}$*Ku70*$^{-/-}$. All MEFs were derived in house from day E13.5 embryos and grown in standard DMEM culture conditions. MEFs were plated (5.0x10^5 cells) onto 10cm dishes, and 24 hours later, MEFs were infected with Adeno-Cre retrovirus at an MOI of 500 to delete endogenous Mre11. MEFs were grown for 3 days after infection and split once before plating for experiments.

Immunofluorescence microscopy

MEF cells were plated and grown on glass cover slips in 6-well dishes, treated with 10Gy irradiation (from a 137-cesium source), and allowed to recover for indicated times post-ionizing radiation. Cells were then fixed in 3% paraformaldehyde/2% sucrose solution for 10 minutes at room temperature, followed by a permeabilization step in Triton-X-100 solution (0.5% Triton X-100; 20mM HEPES, pH 7.4; 50mM sodium chloride; 3mM magnesium chloride; and 300mM sucrose) for 3 minutes on ice. For KU foci, the permeabilization step was coupled with a pre-extraction step in which 0.3mg/mL RNAse A (Roche Diagnostics) was added to the Triton-X-100 solution. Fixed cells were incubated with the following antibodies in 5% goat serum/PBS solution for 20 minutes at 37 °C: anti-KU70 monoclonal rabbit antibody at 1:100 (Cell Signaling, 4588s); anti-γH2AX monoclonal mouse at 1:1,000 (EMD Millipore, 05-636); and anti-53BP1 at 1:200 (Novus Biologicals, NB100-904). Cells were then incubated with goat anti-mouse

Alexa Fluor 488 antibody (1:800, Molecular Probes, A11001) or goat anti-rabbit Alexa Fluor 594 (1:800, Molecular Probes, A11012) antibody in 5% goat serum in PBS for 20 minutes at 37 °C. Stained coverslips were mounted with Prolong Gold anti-fade reagent with DAPI overnight at room temperature.

Images were acquired using the Olympus BX61 microscope and the FISHview Software (Applied Spectral Imaging). For KU70 foci, Mean Fluorescence Intensity was quantified using ImageJ64 software. Nuclei were selected using the freehand selection tool. Measurements were obtained for the following parameters: area, mean, standard deviation, mode, minimum, maximum, integrated density, median, and raw integrated density. The Mean Fluorescence Intensity was obtained using data from the "mean" category and standard error of the mean (SEM) was then calculated. Three independent experiments were conducted and analyzed for all foci experiments.

Laser microirradiation and live-cell imaging

MEFs were transfected with MDC1-BRCT-eGFP, KU70-GFP, KU80-GFP, or GFP-NBS1 in glass-bottomed culture dishes (MatTek Corporation). Laser microirradiation damage was induced with an IX71 Microscope (Olympus) coupled with the MicroPoint Laser Illumination and Ablation System (Photonic Instruments). Images were taken with the same microscope using CellSense software (Olympus) at the indicated times post-microirradiation damage.

Western blotting

Cells were harvested and lysed in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% C24H39O4Na, 0.1% SDS, and 50mM Tris-HCl, pH 8.0), resolved by SDS-PAGE, and transferred under standard conditions. The following primary antibodies were used: MRE11 (Cell Signaling), RAD50 (Bethyl Laboratories), NBS1 (Novus Biologicals), KU70 (Cell Signaling), and TOPI (BD Biosciences). Secondary antibodies for western blots were IRDye-conjugated goat anti-rabbit or anti-mouse (Li-Cor Biosciences). Quantification of band intensities was performed with Li-Cor Odyssey 2.1 software.

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Chapter III

The MRE11 C-Terminus Regulates the Catalytic Activity of CDK2 in an S-Phase Checkpoint Pathway†

Abstract

DNA double-strand breaks (DSBs) are dangerous lesions that if not properly repaired lead to mutations, chromosomal aberrations, and tumorigenesis. The MRE11/RAD50/NBS1 (MRN) complex, a sensor of DSBs, facilitates activation the ataxia-telangiectasia mutated (ATM) kinase, which in turn has roles in initiating DNA repair pathways, cell cycle checkpoints, and in some cases, apoptosis or senescence. Hypomorphic mutations in *MRE11* cause the rare disease ataxia-telangiectasia like disorder (ATLD), which presents clinically with cerebellar degeneration, ataxia, and occasionally, cancer predisposition. *MRE11*^{ATLD1} was the first *MRE11* patient allele described and is caused by C-terminal truncation of the MRE11 protein. *MRE11*^{ATLD1} patient cells have low levels of the MRN complex, defective ATM signaling, radio-sensitivity, and genomic instability. Likewise, a

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MJM contributed to Figures 3.1, 3.2E, 3.3, 3.4A, D, and E, 3.5-3.7, and all Supplementals
TAF contributed to Figures 3.2A-D
JB contributed to Figure 3.4B-C, G, and F

Mre11$^{ATLD1/ATLD1}$ mouse model led to decreased maternal embryonic viability, ATM signaling defects, and chromosomal aberrations. It is not known if the defects found in *MRE11*^{ATLD1} patient and *Mre11*$^{ATLD1/ATLD1}$ mouse cells are due solely to low levels of the MRN complex, among other functions, or if loss of the MRE11 C-terminus contributes. We previously described a role for the MRE11 C-terminus in 2012, when our lab discovered that MRE11 interacts with cyclin dependent kinase 2 (CDK2). We demonstrated that the MRE11 C-terminus is both required and sufficient for interaction with CDK2, and that this interaction has roles for homologous recombination in normally dividing cells.

To further uncover functions of the MRE11 C-terminus, and to investigate roles for the MRE11-CDK2 interaction in the DNA damage response, we utilized MRE11-deficient, MRE11-nuclease deficient, and MRE11 C-terminal truncated expressing cells in experiments with DSBs induced by ionizing radiation (IR). We found that IR caused dissociation of the MRE11-CDK2 interaction in an ATM-dependent manner, as well as a reduction in activating phosphorylation of CDK2 on Threonine 160 and in CDK2 catalytic activity. Interestingly, genetic disruption of the MRE11-CDK2 interaction in MRE11-deficient and MRE11 C-terminal truncated expressing cells also resulted in reduced basal levels of CDK2 catalytic kinase activity and pCDK2^{Thr160} levels, mimicking IR-induced disruption of the interaction. Deficiency of MRE11 nuclease activity did not affect CDK2 activity levels. This data implies that MRE11, specifically its C-terminus, has roles in maintaining normal levels of CDK2 activity. Interestingly, MRE11 C-terminal truncation did not cause defects in S-phase checkpoint functions, suggesting that

previously described defective S-phase checkpoint phenotypes in *MRE11*^{ATLD1} patient and *Mre11*$^{ATLD1/ATLD1}$ mouse cells are due to low levels of the MRN complex, and not due to loss of the MRE11 C-terminus alone. Finally, we found that the MRE11-CDK2 interaction is not disrupted after IR in two human cancer cell lines, U2OS and HeLa. From our studies, we conclude that the MRE11-CDK2 interaction has roles in controlling CDK2 activity important for initiation of the S-phase checkpoint, and this pathway appears to be altered in cancer cells. Thus, MRE11 is a critical component of the cell cycle machinery through its interaction with CDK2.

Introduction

DSBs are among the most toxic forms of DNA lesions and are caused by exogenous sources such as chemicals, ionizing irradiation, and reactive oxygen species or can arise as a result of endogenous processes such as during replication[1]. Unrepaired DSBs lead to mutations and chromosomal aberrations that are essential in the transformation of normal cells into cancer cells[2, 3]. Thus, it is imperative that DSBs are fixed. The two main DSB repair pathways are non-homologous end-joining (NHEJ), a cut-and-paste method of DNA repair, and homologous recombination (HR), which repairs DSBs using a template sister chromatid[4]. Additionally, to combat the threat to the genome posed by DSBs, organisms have evolved complex networks of signaling pathways, termed the DNA Damage Response (DDR), that sense, signal, and repair DNA lesions[5].

MRN is a sensor protein complex important in recognizing and binding DSBs and is required for the recruitment and activation of the ATM kinase, the master regulator of the DDR[6-12]. Once activated, ATM phosphorylates a variety of proteins involved in DNA repair, cell cycle checkpoint control, and apoptotic responses, including checkpoint kinase 2 (CHK2), cellular tumor antigen p53 (p53), breast cancer type 1 susceptibility protein (BRCA1), H2A histone family, member X (H2AX), structural maintenance of chromosome protein 1 (SMC1), ARTEMIS, and Nijmegen breakage syndrome 1 (NBS1)[13-19]. Phosphorylation of these and other substrates by ATM initiates cell cycle arrest at G1/S, intra-S, and G2/M checkpoints and promotes DNA repair. Mutations in the *ATM* gene are responsible for the rare autosomal recessive disorder ataxia-telangiectasia (A-T),

which is characterized by cerebellar degeneration, immunodeficiency, and an increased risk of cancer[20]. Cells from individuals with A-T exhibit defects in DNA damage-induced checkpoint activation, radiation hypersensitivity, and an increased frequency of chromosomal aberrations[21].

The functional relationship between ATM and MRN first became clear when it was discovered that patients with mutations in the MRN complex had similar clinical phenotypes as those found in A-T patients[22]. Loss of any of the individual components of the MRN complex results in embryonic lethality in mice[23-25]. Because no known human disease alleles are null, MRN patient mutations must be hypomorphic to preserve functions of the MRN complex necessary for embryonic development. Hypomorphic mutations in the *NBS1* gene cause the disease Nijmegen breakage syndrome (NBS) which is characterized by cancer predisposition, immunodeficiency, and microcephaly, while hypomorphic mutations in *RAD50* cause NBS-like disorder[26, 27]. Partial loss-of-function mutations in *MRE11* cause ataxia-telangiectasia-like disorder (ATLD)[22]. Interestingly, ATLD patient cells harbor low levels of mutant MRE11 protein, markedly reduced activation of ATM by DNA DSBs, and reduced ATM-dependent phosphorylation of downstream substrates[28, 29]. This observation supports the notion that MRN is required for optimal ATM activation following DNA DSB induction.

Thus far, several ATLD patient alleles have been identified. ATLD patient phenotypes include progressive cerebellar degeneration resulting in ataxia, developmental delay, and a varied predisposition to cancer[29]. *MRE11*^{ATLD1} was

the first reported ATLD patient allele and contains a nonsense mutation that truncates 76 amino acid residues from the C-terminus of MRE11[30]. *MRE11*^{ATLD1} patient cells have low levels of the MRN complex, are radiosensitive, and have S-phase checkpoint defects. In 2003, mice with the *Mre11*^{ATLD1} allele (termed *Mre11*$^{ATLD1/ATLD1}$) were derived to gain more insight into the *in vivo* functions of the MRN complex[31]. The mutation had a severe maternal effect on embryonic viability, and also resulted in low levels of the MRN complex and impaired ATM signaling, cell cycle checkpoint defects, and pronounced chromosomal instability in mouse-derived cells[31]. The mice, however, were not prone to cancer, suggesting that the defective cell cycle checkpoints and chromosomal instability were insufficient to initiate tumorigenesis[31]. While both *MRE11*^{ATLD1} patient and *Mre11*$^{ATLD1/ATLD1}$ mouse cells exhibit similar phenotypes, it is unclear if these phenotypes are due to low levels of the MRN complex or due to loss of the MRE11 C-terminus specifically.

Interestingly, our lab discovered and published in 2012 that the MRE11 C-terminus is required for interaction with CDK2, the kinase responsible for cell cycle progression in S-phase[32]. Binding of CDK2 with its regulatory partner, Cyclin A, promotes S-phase progression, the phase of the cell cycle where DNA replication takes place. Problems during the replication process initiate the intra-s-phase checkpoint, which exists to delay progression through S-phase, allowing time for the DNA to be repaired before mitosis[33]. Buis et al., found that interaction of MRE11 with CDK2 is important for regulating homologous recombination in normally cycling cells[32].

This brought us to the question of how the MRE11-CDK2 interaction might function during the DDR in response to DSBs. Taking all this knowledge together, we hypothesized that the MRE11 C-terminus, through interaction with CDK2, is a component of the intra-S-phase checkpoint. To test our hypothesis, we utilized mouse embryonic fibroblast (MEF) cells in our lab harboring a conditional allele, which upon addition of Cre-adenovirus, confers MRE11-deficiency or MRE11-nuclease deficiency[24]. Additionally, we generated an *Mre11*^{ATLD1}-mutant expressing cell line from a cDNA in which endogenous MRE11 is deleted via the Cre/LoxP system[34]. We utilized these MRE11-deficient and MRE11 C-terminal truncated expressing cells as tools to study the MRE11-CDK2 interaction *in vitro*, with the addition of ionizing radiation to experimentally induce DSBs. Through our studies, we have identified novel functions for the MRE11-CDK2 interaction in cell cycle checkpoint control in normal cells, which appears to be altered in two cancer cell lines. First, we observed that IR-induced DNA damage led to disruption of the MRE11-CDK2 interaction in an ATM-dependent manner, as well as a reduction in CDK2 catalytic activity levels. Interestingly, genetic disruption of the interaction in MRE11-deficient and MRE11 C-terminal truncated expressing cells also resulted in a reduction of CDK2 catalytic activity, mimicking IR-induced disruption of the interaction and this was independent of MRE11 nuclease activity. Phosphorylation of CDK2 on Threonine 160, a phosphorylation event that promotes CDK2 activity, was decreased by both IR and by genetic disruption of the MRE11-CDK2 interaction. These data imply that MRE11 has roles in controlling CDK2 activity through its C-terminus. Because a decrease in CDK2 activity is important for

stalling progression through S-phase, we hypothesize that IR-induced disruption of the MRE11-CDK2 interaction is a component of initiating this checkpoint response. The decreased basal levels of CDK2 activity observed in the absence of the MRE11 C-terminus suggests that this checkpoint response is already activated in these cells. Indeed, we observed slow proliferation in the MRE11 C-terminal truncated expressing cells independent of dramatic genomic instability, a characteristic of MRN-deficiency. Intriguingly, the MRE11-CDK2 interaction was not disrupted following IR treatment in two human cancer cells lines, U2OS and HeLa, implying the proposed S-phase checkpoint response initiated by disruption of the interaction is defective in these cancer cells. Next, we analyzed S-phase checkpoint responses to IR in MRE11-deficient and MRE11 C-terminal truncated expressing cells. MRE11-deficiency resulted in defects in S-phase cell cycle checkpoint function while MRE11 C-terminal truncation did not cause a defect, suggesting that previous S-phase checkpoint defects described in *MRE11*^{ATLD1} patient and *Mre11*$^{ATLD1/ATLD1}$ mouse cells are due to low levels of the MRN complex and defective ATM signaling, and do not appear to be caused by MRE11 C-terminal truncation alone. Taken together, our research has uncovered that MRE11, specifically the C-terminus, has roles in controlling CDK2 catalytic activity important for S-phase checkpoint function through interaction with CDK2.

Results

Generation of mutant MRE11-expressing cell lines

Past studies investigating the role of the MRN complex, specifically MRE11 and its C-terminal domain, utilized *MRE11*^{ATLD1} patient and *Mre11*$^{ATLD1/ATLD1}$ mouse cells which result in not only C-terminal truncation, but also low levels of the entire MRN complex and defects in ATM signaling[30, 31]. These studies raise the question of whether phenotypes previously describing an S-phase checkpoint defect in patient and mouse cells are truly due to functions of the MRE11 C-terminus or in fact to low levels of the MRN complex. To further interrogate these mysteries, we utilized a mouse system previously generated in our lab in which mice lack MRE11 entirely, express an MRE11 nuclease-deficient allele, or express a cDNA for the *Mre11*^{ATLD1} mutation[24, 34].

To bypass the embryonic lethality conferred by MRE11-deficiency or defective MRE11-nuclease activity, we utilized immortalized MEFs from mice harboring a conditional *Mre11* allele that is inactivated through Cre/LoxP-mediated recombination[24]. In this process, regions of the critical exon 5 of *Mre11* are excised, resulting in MRE11 protein deletion. Exposure of Cre-adenovirus to *Mre11*$^{Cond/-}$ MEFs in cell culture results in *Mre11*$^{-/-}$ cells, which also causes deficiency of the other components of the MRN complex (RAD50 and NBS1), most likely due to instability of the complex when MRE11 is absent[24]. Additionally, we used an allele containing a targeted single amino acid change (*Mre11*^{H129N}) that eliminates both the endo- and exo-nuclease activities of MRE11 without disrupting MRN complex formation or its ability to sense DSBs and activate ATM[24].

To study the effects of C-terminal truncation of MRE11, we stably transfected *Mre11*$^{Cond/-}$ MEFs with pEF6-Mre11 constructs expressing the *Mre11*^{ATLD1} mutant from cDNA[34]. Clones were isolated, and cells exposed to cre-adenovirus in culture to delete endogenous MRE11 protein. The clone used in this study was chosen because it expresses physiologic levels of the MRN complex[34].

For experiments, MEF cells were split, cre-adenovirus was added, and after 5 days in cell culture, cells were plated for experiments (Figure 3.1A). Thus, genotypes used in this study are *Mre11*$^{Cond/-}$, *Mre11*$^{-/-}$, *Mre11*$^{H129N/-}$, *Mre11*$^{Cond/-}$ + *Mre11*^{ATLD1} cDNA, and Mre11$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing MEFs (Figure 3.1B). *Cdk2*$^{-/-}$ and *Atm*$^{-/-}$ MEF cells were often used as controls in several experiments. Loss of MRE11 or the MRE11 C-terminus was not found to affect levels of CDK2 protein. Likewise, *Cdk2*$^{-/-}$ cells have normal levels of MRE11 protein (Figure 3.1C). Of note, all MEFs used in this study are SV40 large T-antigen immortalized.

It is known that the MRN complex is required for recruitment and activation of ATM to DSBs[6-8, 12, 35, 36]. The activation of ATM is important during the DNA damage response as ATM phosphorylates and activates many proteins, which have roles in facilitating DNA repair, cell cycle checkpoints, and apoptosis[37]. Thus, I measured the ability of *Mre11*$^{-/-}$ and *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing cells to activate ATM after DNA DSB induction by examining phosphorylation of the ATM substrates, KRAB [Kruppel-associated box domain]-associated protein 1 (KAP1) and CHK2. I induced DSBs with 10Gy ionizing

radiation and harvested cells 30 minutes post-treatment. As expected, *Mre11*$^{-/-}$ cells exhibited defects in ATM signaling activation[24]. I confirmed that C-terminal truncation of MRE11 mimics controls and does not confer defects in ATM signaling, as previously described by our lab[34]. Phosphorylation of KAP1 looked similar to controls, as did phosphorylation of CHK2 (as detected by band shift after IR treatment) (Supplemental Figure 3.1). Thus, any phenotypes found in the *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} expressing cells can be attributed to C-terminal truncation of MRE11 and not due to low levels of the MRN complex or defects in ATM signaling. Additionally, I examined localization of MRE11 and CDK2 in MRE11 C-terminal truncated expressing cells in biochemical fractionation experiments. Importantly, loss of the MRE11 C-terminus did not cause a defect in localization of MRE11 to chromatin as compared to its matched control. CDK2 localization was restricted to the cytoplasmic and nuclear fractions in both control and *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing cells (Supplemental Figure 3.2).

The MRE11-CDK2 interaction is disrupted after DNA damage induced by ionizing radiation

Previously, our lab discovered that the DNA repair protein MRE11 interacts with the cell cycle kinase CDK2 and found that this interaction is important for regulating homologous recombination in normally dividing cells[32]. MRE11 interaction with CDK2 is necessary for CDK2 to phosphorylate DNA endonuclease RBBP8 (CTIP). Phosphorylated CTIP can then bind BRCA1, and this tri-part interaction between MRN, CTIP, and BRCA1 facilitates resection in S and G2

phases of the cell cycle[24, 38-41]. This data shows that MRE11's roles are not restricted to the DNA damage response, but that it also functions in cells under normal conditions through its interaction with CDK2.

We next set out to investigate roles for the MRE11-CDK2 interaction in the DNA damage response. First, we examined the interaction in immortalized *Mre11*$^{Cond/-}$ MEFs under normal conditions or treated with 10Gy ionizing radiation to induce DSBs. Todd Festerling, a former graduate student in the lab, performed these initial immunoprecipitation experiments. Cells were harvested at both early and late timepoints in a time-course of 15, 30, and 60 minutes after IR treatment. Co-immunoprecipitations were performed by immunoprecipitating CDK2 and blotting for the interaction with anti-MRE11 antibody. Cyclin A antibody was used to confirm successful pulldown with CDK2. The MRE11-CDK2 interaction was un-altered at all time-points under normal conditions but was not detected post-IR treatment (Figure 3.2A). Interestingly, Cyclin A still immunoprecipitated with CDK2 after IR. Thus, at both early and late timepoints, DNA DSBs cause the MRE11-CDK2 interaction to dissociate in transformed MEFs. However, DNA damage does not alter the interaction between CDK2 and Cyclin A.

Next, we aimed to confirm the reciprocal co-immunoprecipitations of MRE11 and CDK2. Therefore, in transformed MEFS, we immunoprecipitated MRE11 and blotted for CDK2. NBS1 was blotted to confirm successful pulldown with MRE11. MEF cells were either mock-treated or treated with 10Gy IR and harvested 30 minutes post-treatment. As seen previously, the MRE11-CDK2

interaction was detected in normal cells, but not in irradiated cells, suggesting that DNA damage leads to disruption of this interaction (Figure 3.2B).

Although we have confirmed this interaction is present in transformed MEFs, we thought it would be equally important to investigate whether the MRE11-CDK2 interaction also exists in other cell types. Therefore, we performed co-immunoprecipitation experiments in primary MEFs and primary human cells. For these experiments, we immunoprecipitated MRE11 and blotted for CDK2. NBS1 was blotted to confirm that MRE11 was indeed immunoprecipitated. The cells were either mock-treated or treated with 10Gy IR and harvested 60 minutes post-treatment. Here, we show that the MRE11-CDK2 interaction is present in both primary MEFs and primary human cells under normal conditions and DNA damage by IR disrupts the MRE11-CDK2 interaction in both cell types (Figures 3.2C-D). This data implies that the MRE11-CDK2 interaction exists in several cell types and that disruption by IR appears to be universal in the cell types that we investigated.

We have previously shown that the MRE11 C-terminus is required for interaction with CDK2[32]. To further interrogate the requirements of MRE11 protein for its interaction with CDK2, I performed immunoprecipitations in cells lacking the MRN complex or the MRE11 C-terminus. In these experiments, I immunoprecipitated CDK2 in either *Mre11*$^{-/-}$ or *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing MEFs compared to their matched control cell lines and blotted for MRE11 via western blot analysis. CDK2 was probed to confirm successful CDK2 pulldown. MRE11 co-immunoprecipitated with CDK2 in *Mre11*$^{Cond/-}$ MEFs, but not in *Mre11*$^{-/-}$ MEFs (Supplemental Figure 3.3A). This data demonstrates that the

MRE11-CDK2 interaction does not occur in MRE11-deficient cells, as expected. Likewise, CDK2 interacted with endogenous full-length MRE11 in *Mre11*$^{Cond/-}$ + Mre11^{ATLD1} cDNA expressing cell lines, but not with the truncated, C-terminal deleted version of MRE11. There was no interaction present in *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing cells, where both endogenous MRE11 and the MRE11 C-terminus are deleted (Supplemental Figure 3.3B). These data imply that, indeed, the MRE11 C-terminus is required for interaction with CDK2. Thus, MRE11-deficiency and MRE11 C-terminal truncation genetically disrupt the MRE11-CDK2 interaction and can be used as tools in our studies to further investigate roles for the interaction during the DNA damage response.

Next, we were interested in further examining the requirement of the MRE11 C-terminus for its interaction with CDK2 after ionizing radiation. To this end, I transfected 293T cells with a FLAG-tagged MRE11 C-terminal construct (denoted C76) and an empty vector construct (EV) as a negative control. After transient transfection, cells were either mock-treated or treated with 10Gy ionizing radiation and harvested 30 minutes after treatment. Anti-FLAG antibody was used to detect expression of the MRE11 C-terminus and GAPDH was used as a loading control (Figure 3.2E). I observed robust expression of C76 and no change in its expression after ionizing radiation treatment by western blot analysis.

Next, I performed co-immunoprecipitation experiments in 293T cells expressing EV or C76 constructs. Cells were either mock or IR treated and harvested 30 minutes post-IR. The FLAG-tagged MRE11 C-terminus was immunoprecipitated with anti-FLAG antibody. Western blots were probed with

either anti-FLAG antibody to confirm pulldown or anti-CDK2 antibody to examine co-immunoprecipitation. As expected, I found that the FLAG-tagged MRE11 C-terminus is sufficient to pulldown CDK2[32]. However, after IR treatment, CDK2 no longer co-immunoprecipitated with the MRE11 C-terminus (Figure 3.2F). This data implies that the MRE11 C-terminus contains the faculties necessary for control of the IR-dependent disruption of the MRE11-CDK2 interaction.

Disruption of the MRE11-CDK2 interaction after DNA damage is ATM-dependent

ATM kinase is the master regulator of the DNA damage response. Its activation is important for initiating cellular responses to DSBs, including DNA repair, cell cycle checkpoints, transcription, and cell death[1]. Because of ATM's roles in response to DSBs, we hypothesized that ATM may regulate the dissociation of the MRE11-CDK2 interaction after DNA damage. To test this hypothesis, I co-immunoprecipitated CDK2 from control and *Atm*$^{-/-}$ MEFs and analyzed the interaction status by blotting for MRE11. Cells were either mock-treated or treated with 10Gy IR and harvested 30 minutes post-treatment. I found that the MRE11-CDK2 interaction was disrupted after treatment with IR in control cells, but not in *Atm*$^{-/-}$ cells (Figure 3.3A).

Next, I analyzed the MRE11-CDK2 interaction in transformed *Mre11*$^{Cond/-}$ MEFs, either untreated or treated with KU-55933 (a potent and selective inhibitor of ATM) in response to IR (10Gy and 30-minute recovery)[42]. Cells were treated

with 10μM ATM inhibitor for 1 hour before IR treatment. CDK2 was immunoprecipitated from cells and western blots were probed with anti-MRE11 antibody. Like the co-immunoprecipitations in *Atm*$^{-/-}$ cells, I found that the MRE11-CDK2 interaction was not disrupted when cells were treated with the ATM inhibitor after IR (Figure 3.3B). Together, these results suggest that the dissociation of the MRE11-CDK2 interaction after DNA damage is dependent on the ATM kinase.

Ionizing radiation and genetically-induced disruption of the MRE11-CDK2 interaction cause reduction in phosphorylation of CDK2 on Threonine 160 and in CDK2 catalytic activity

Previous studies have reported that DNA damage induced by ionizing radiation causes reduction in CDK2 catalytic activity and that this reduction in activity is important for initiating the S-phase checkpoint, in which S-phase progression is halted, allowing for DNA to be repaired before the cell divides in mitosis[43, 44]. In fact, several S-phase checkpoint pathways have been described. One pathway involves ATM-dependent phosphorylation of CHK2, degradation of the phosphatase CDC25A, inhibitory phosphorylation of CDK2, and a reduction in CDK2 activity which inhibits chromatin-associated loading of CDC45, a member of the pre-replication complex, thus stalling replication and progression of S-phase[43, 45]. Another S-phase checkpoint pathway previously described involves ATM-mediated phosphorylation of NBS1 and SMC1[46]. Mutations of ATM phosphorylation sites on NBS1 (Serine 343) and SMC1 (Serines 957 and 966) result in S-phase checkpoint defects[46]. It is

possible that other mechanisms for inducing the S-phase checkpoint through reduction in CDK2 activity exist. Therefore, we hypothesized that dissociation of the MRE11-CDK2 interaction causes reduction in CDK2 activity after IR, implying that the MRE11-CDK2 interaction is a component of an S-phase checkpoint pathway. To test this hypothesis, I performed an *in vitro* CDK2 kinase assay to examine the enzymatic catalytic activity of CDK2 in conjunction with association/dissociation of MRE11-CDK2 interaction in a time-course after IR. In the kinase assay, CDK2 antibody was used to isolate CDK2 complexes which are subsequently incubated with histone H1 protein substrate in the presence of radioactive ATP. After the termination of the kinase reaction, the amount of radioactively labeled substrate, which reflects the extent of kinase activity, can be visualized on a polyacrylamide gel and exposed to film[47].

I simultaneously examined CDK2 activity at the same time as MRE11-CDK2 interaction 15, 30, 60, and 120 minutes after ionizing radiation treatment (Figure 3.4A). CDK2 activity was highest in normal cells and this corresponded to an intact MRE11-CDK2 interaction. After IR, I observed a steady decrease in CDK2 kinase activity and disruption of the MRE11-CDK2 interaction. KAP1 phosphorylation was measured to analyze ATM activation kinetics in the cells in response to IR. It is important to note that there was intermittent variability of either the interaction status of MRE11-CDK2 or reduction in kinase activity at 15 minutes post-IR treatment. This could be due to the technical difficulty of harvesting cells at such an early time-point after IR. In the figure presented here, I did not observe dissociation of the MRE11-CDK2 interaction until 30 minutes after IR, whereas, in

other experiments, we have seen dissociation as early as 15 minutes. Thus, our data cannot confirm whether the MRE11-CDK2 dissociation after IR causes CDK2 activity to decrease and to initiate the S-phase checkpoint. Instead, our data implies that ionizing radiation causes a reduction of CDK2 kinase activity and disruption of MRE11-CDK2 interaction in cells at the same time.

Phosphorylation of CDK2 on Threonine 160 by CDK-activating (CAK) kinases promotes CDK2 activity, along with binding to Cyclin A or E[48, 49]. Jeffrey Buis, a former post-doctoral fellow in the lab, examined pCDK2^{Thr160} in control (*Atm*$^{+/+}$) cells on a time-course of 15, 30, 60, and 240 hours after 10Gy IR treatment. Phosphorylation of the histone variant H2AX (denoted as γH2AX) was used as a marker for activation of the DNA damage response[14]. We observed lower levels of pCDK2^{Thr160} in cells at all time-points after IR and most strikingly at 15 and 30 minutes post-DSB induction (Figure 3.4B). This data mirrors the reduction in CDK2 activity we observed in control cells after IR and offers a mechanism for reduction in CDK2 catalytic activity. Jeffrey Buis next examined phosphorylation of CDK2 on Threonine 160 in *Atm*$^{-/-}$ cells on a time course of 15, 30, 60, and 120 minutes after IR. Notably, we observed a delayed response in phosphorylation of H2AX after IR in *Atm*$^{-/-}$ cells as compared to controls. Moreover, IR did not cause a decrease in phosphorylation of CDK2 on Threonine 160 in *Atm*$^{-/-}$ cells after IR (Figure 3.4C). This data suggests that inhibition of CDK2 activity via dephosphorylation of Threonine 160 on CDK2 after DNA damage is an ATM-dependent process.

We next investigated how loss of the MRN complex or loss of the MRE11 C-terminus effects levels of CDK2 activity. Thus, I conducted *in vitro* CDK2 kinase assays in *Mre11*$^{Cond/-}$, *Mre11*$^{-/-}$, *Mre11*$^{Cond/-}$ + *Mre11*^{ATLD1} cDNA, and *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing MEFs. CDK2 was purified from lysates using anti-CDK2 antibody and *Cdk2*$^{-/-}$ MEFs were used as a negative control. Inputs show successful deletion of endogenous MRE11 using adeno-cre virus. Interestingly, loss of MRN and the MRE11 C-terminus both resulted in decreased basal levels of CDK2 catalytic activity compared to matched controls (Figure 3.4D). At least three independent experiments were conducted, and activity was quantified using ImageJ software. Data is graphed by pixel density (Figure 3.4E). Additionally, Jeffrey Buis analyzed the impact of MRE11 nuclease deficiency on CDK2 catalytic activity, and saw no effect, suggesting that the nucleolytic activities of MRE11 are not important for control of CDK2 activity (Figure 3.4F). From this data, we conclude that MRE11, specifically the C-terminus, is required for maintaining normal levels of CDK2 catalytic activity.

Jeffrey Buis next analyzed basal levels of phosphorylation of CDK2 on Threonine 160 in *Mre11*$^{Cond/-,}$ *Mre11*$^{-/-}$, *Mre11*$^{H129N/-}$, and *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} expressing cells (Figure 3.4G). As expected, we observed lower levels of pCDK2^{Thr160} in MRE11-null and MRE11 C-terminal truncated mutant MEFs, mirroring our previous observations of reduced basal CDK2 activity in these mutant cells. Loss of MRE11 nuclease activity did not impact pCDK2^{Thr160} levels. Taken together, we demonstrate that genetic disruption of the MRE11-CDK2 interaction mimics IR-induced disruption of the interaction, both of which result in lower

catalytic CDK2 activity and pCDK2^{Thr160} levels. This data implies that the MRN complex, specifically the MRE11 C-terminus, regulates levels of CDK2 activity through phosphorylation of CDK2 on Threonine 160, independent of MRE11 nuclease activity.

MRE11 C-terminal truncation results in slow proliferation equivalent to that of MRE11-deficiency, but only a mild genomic instability phenotype

Because CDK2 is the central cell cycle kinase that regulates S-phase progression, I decided to investigate whether lower basal CDK2 activity levels impact proliferation rates in cells lacking the MRE11 C-terminus. Loss of the MRN complex has previously been shown to cause proliferation defects in cells, and so I utilized this cell line as a control for slow cell growth, along with *DNA ligase IV*$^{-/-}$ and *Atm*$^{-/-}$ MEFs[24, 50, 51]. Consequently, I examined proliferation of all genotypes over the course of a five-day period in cell culture. Surprisingly, I observed slow cell growth in the MRE11 C-terminal truncation mutant, which was comparable to the slow growth observed in MRE11-null cells (Figure 3.5A). Importantly, the addition of cre-adenovirus to *Mre11*$^{+/+}$ cells did not affect cell proliferation, demonstrating that the virus did not have any off-target effects that might confound our results (Supplemental Figure 3.4). This data complements our earlier experiments demonstrating equally low levels of CDK2 catalytic activity in both MRE11-mutants. This brought us to two possibilities: 1) Genetic disruption of the MRE11-CDK2 interaction causes lower levels of CDK2 activity, mimicking initiation of the S-phase checkpoint and leading to slower proliferation rates in the

cells or 2) The slower proliferation rates are not due to disruption of the MRE11-CDK2 interaction, but rather to increased DNA damage in the cells.

Next, I tested the hypothesis that the slower proliferation rate in *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing cells is due to genomic instability. First, cells were stained with the fluorescent intercalating agent Propidium Iodine (PI), which binds to DNA, to evaluate DNA content in cell cycle analysis via flow cytometry. Cell cycle analysis revealed normal cell cycle profiles for control cell lines, as expected. MRE11-deficiency, on the other hand, resulted in a small sub-G1 peak that most likely signifies DNA fragmentation and loss of DNA content due to dramatic genomic instability. MRE11 C-terminal truncation did not result in a sub-G1 peak, although an increase of cells in G1 and a decrease of cells in S-phase was observed (Figure 3.5B). This data hints at a severe genomic instability phenotype in the absence of the MRN complex, but not in the absence of the MRE11 C-terminus.

Cytogenetics, or the analysis of chromosomes, is the gold standard for the identification of the chromosomal anomalies that define genomic instability. To examine chromosome anomalies, cells were treated with the drug Colcemid which prevents spindle formation during mitosis, causing cells to arrest in mitosis so that chromosomes can be separated for cytogenetic analysis. The bar graph shows the number of chromosomal anomalies per metaphase with the types of anomalies listed in the key. The table shows proportions of anomalies scored for each genotype (Figure 3.5C). The anomalies identified include fragments, breaks, dicentrics, Robertsonian translocations, circular chromosomes, and radial

structures (Supplemental Figure 3.5). Loss of MRE11, as expected, led to dramatic genomic instability, as seen previously[24]. ATM-deficiency also led to increased chromosomal anomalies, but not to the same extent as MRE11-deficiency, consistent with studies demonstrating that MRE11-defiency leads to the more severe phenotype of embryonic lethality in mice, while ATM-deficiency does not[24, 51]. MRE11 C-terminal truncation resulted in an increase in chromosomal anomalies compared to its matched control, but this phenotype was not as severe as the genomic instability observed in either MRN or ATM deficiencies. From this data, we can conclude that the dramatic genomic instability due to loss of the MRN complex is a major contributor to slow proliferation and low levels of CDK2 activity in these cells. However, MRE11 C-terminal truncation, which supports ATM activation and MRN complex formation, exhibited only a mild genomic instability phenotype not likely severe enough to cause the slow growth and low CDK2 activity levels observed in these cells[34]. Together, these studies support the notion that genetic disruption of the MRE11-CDK2 interaction due to loss of the C-terminus mimics IR-induced disruption of the interaction, constitutively activating the S-phase checkpoint in these cells, leading to lower CDK2 activity levels and slow proliferation.

Examination of inhibitory phosphorylation of CDK2 on Tyrosine 15 and inhibition of CDK2 kinase activity after ionizing radiation treatment in MRE11-mutants

Previous studies have shown that *MRE11*^{ATLD1} patient cells and *Mre11*$^{ATLD1/ATLD1}$ mouse cells have defects in cell cycle checkpoints, including the intra-s-phase checkpoint[22, 31]. Because we found that the MRE11-CDK2 interaction is disrupted after IR in an ATM-dependent manner, and CDK2/Cyclin A regulate progression in S-phase of the cell cycle, we set out to analyze the effects of IR-induced DNA damage on S-phase checkpoint functions in the absence of either the MRN complex or the MRE11 C-terminus.

I utilized MRE11-deficient and MRE11 C-terminal truncated expressing cells, which genetically disrupt the MRE11-CDK2 interaction, in our experiments. As mentioned previously, studies have reported that inhibition of CDK2 activity after IR is important for induction of the S-phase checkpoint, and this pathway is deregulated in ATM-deficient cells[43, 44]. Therefore, I began by measuring catalytic CDK2 activity in control *Mre11*$^{Cond/-}$ and *Atm*$^{-/-}$ MEFs in response to DNA damage (Figure 3.6A). CDK2 was immunoprecipitated and kinase assay reactions performed from cells mock-treated or IR-treated and harvested 30 minutes post-treatment. I observed inhibition of CDK2 activity after IR in control cells, but not in *Atm*$^{-/-}$ cells. This data confirms that S-phase checkpoint induction by inhibition of CDK2 activity after IR is ATM-dependent, as previously described[43]. Next, I examined CDK2 catalytic activity in response to IR in *Mre11*$^{Cond/-}$, *Mre11*$^{-/-}$, *Mre11*$^{Cond/-}$ + *Mre11*^{ATLD1} cDNA, and *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing MEFs

(Figure 3.6B). 10Gy IR was used to induced DSBs and cells were harvested 30 minutes after treatment. Control cells showed a reduction in CDK2 catalytic activity after IR. However, *Mre11*$^{-/-}$ cells, like *Atm*$^{-/-}$ cells, did not inhibit CDK2 activity following DSB induction, while MRE11 C-terminal truncation still allowed for inhibition of CDK2 activity after IR. These data are graphed as a +IR/-IR ratio in which at least 3 independent kinase assays were performed per genotype and quantified using ImageJ software (Figure 3.6C). Our results suggest that inhibition of CDK2 activity after IR depends on full-length MRE11, but not's its C-terminus.

The catalytic activity of CDK2 is regulated in numerous ways, including binding of Cyclins A or E, activating phosphorylation of CDK2 on Threonine 160, or inhibitory phosphorylation of CDK2 on Tyrosine 15 or Threonine 14[52]. Inhibitory phosphorylation of CDK2 plays important roles in regulating the activity of CDK2 during the DNA damage response, whereby inhibitory phosphorylation of CDK2 on Tyrosine 15 and Threonine 14 contributes to inhibition of CDK2 activity, and eventually a halt of S-phase progression[43]. Thus, I examined inhibitory phosphorylation of CDK2 on Tyrosine 15 as a readout for CDK2 catalytic activity in response to ionizing radiation in MRE11-deficient or MRE11 C-terminal truncated cells. As a control experiment, I analyzed levels of pCDK2^{Y15} in control and *Atm*$^{-/-}$ MEFs (Figure 3.6D). Cells were either mock-treated or IR-treated with 10Gy and harvested after 30 minutes post-treatment. CDK2 complexes were immunoprecipitated using anti-CDK2 antibody, and western blots were probed with anti-pCDK2^{Y15} antibody. *Cdk2*$^{-/-}$ cells were used as negative control. As seen previously in the literature, CDK2 was phosphorylated on Tyrosine 15 after IR

(indicating inhibition of CDK2 activity) in control cells, but not in *Atm*$^{-/-}$ cells[43]. Thus, inhibitory phosphorylation on CDK2 Tyrosine 15 is ATM-dependent after IR. Next, I examined pCDK2^{Y15} levels in *Mre11*$^{Cond/-}$ and *Mre11*$^{-/-}$ MEFs before and after IR treatment (Figure 3.6E). *Mre11*$^{-/-}$ cells, similar to *Atm*$^{-/-}$, was not phosphorylated on Tyrosine 15 to the same extent as matched *Mre11*$^{Cond/-}$ controls. This data supports our findings demonstrating that the MRN complex contributes to the inhibition of CDK2 activity after IR and inhibitory phosphorylation on CDK2 Tyrosine 15 after IR provides a mechanism for this. In contrast, cells lacking the MRE11 C-terminus exhibited phosphorylation on CDK2 Tyrosine 15 after IR to the same extent to its matched control (Figure 3.6F). This data implies that the MRE11 C-terminus is not necessary for inhibition of CDK2 activity or inhibitory pCDK2^{Y15} after IR. It should be noted that I attempted to analyze CDC25A in our experiments but was unable to successfully detect its degradation following IR (data not shown).

MRE11-deficient cells display an intermediate radio-resistant DNA synthesis phenotype while MRE11 C-terminal truncated expressing cells inhibit DNA synthesis after ionizing radiation treatment similar to controls

Defects in the intra-s-phase checkpoint result in the inability of cells to reduce their rate of replication after IR. This phenomenon, known as radioresistant DNA synthesis (RDS), was first observed in cells derived from patients with ataxia-telangiectasia, who have mutations in the *ATM* gene[53]. The assay for measuring RDS (known as the RDS assay) is considered the gold standard in the DNA repair

field. In this assay, the rate of replication is measured by incorporation of radioactively labeled thymidine, followed by quantification using a liquid scintillation counter[54]. I performed the RDS assay on our MRE11-mutant genotypes to determine if either loss of the MRN complex or the MRE11 C-terminus cause defects in inhibition of DNA synthesis after IR (Figure 3.7). Cells were treated with 10Gy IR and harvested for the experiment 4 hours post-treatment. *Atm*$^{-/-}$ cells were used as a positive control for the radioresistant DNA synthesis phenotype. At least 3 independent experiments were conducted, and data is graphed as a +IR/-IR ratio. *Atm*$^{-/-}$ MEFs were found to exhibit the classic RDS phenotype whereas control cells displayed a low +IR/-IR ratio, indicating inhibition of DNA synthesis after IR. *Mre11*$^{-/-}$ MEFs displayed an intermediate phenotype, which has been seen previously in hypomorphic ATLD- and NBS-mutated patient cells[44]. This data was surprising, as one might expect MRE11-deficiency to mimic the severe RDS phenotype caused by ATM-deficiency. This data suggests in the absence of MRE11, inhibition of DNA synthesis after IR might become dependent on other DNA repair kinases, such as ATR or DNA-PKCs. Surprisingly, *Cdk2*$^{-/-}$ cells inhibited DNA synthesis after IR comparable to controls, also implying other CDKs might facilitate S-phase checkpoint function in the absence of CDK2. Loss of the MRE11 C-terminus did not cause a defect in inhibition of DNA synthesis after IR. Together, our data demonstrates that the MRN complex has roles for inhibiting DNA synthesis after IR, but not specifically through the MRE11 C-terminus.

Inhibition of CDC45 loading onto chromatin is thought to be important for stalling replication in response to DNA damage as part of an S-phase checkpoint pathway[44, 55, 56]. Therefore, I analyzed loading of the replication factor CDC45 onto chromatin after DSB induction in control cells and cells lacking either the MRN complex or the MRE11 C-terminus. In control cells following IR, I did not observe the expected decrease in loading of CDC45 onto chromatin[44]. I also examined CDC45 chromatin loading on a time-course of several hours after IR in control cells and in MRE11-mutants, but again, no decrease in loading was detected (Supplemental Figures 3.6A-C). Therefore, I did not use chromatin-associated loading of CDC45 as a marker for replication in our experiments.

The MRE11-CDK2 interaction is not disrupted after DNA damage in the human U2OS and HeLa cancer cells lines

It is known that cell cycle checkpoints and DNA repair mechanisms are deregulated in cancer cells, leading to genomic instability and uncontrolled cell growth[33]. To further understand roles for the MRE11-CDK2 interaction in cancer, I performed co-immunoprecipitations after DNA damage in a panel of cancer cells lines (Figure 3.8). Control MEFs, HeLa (human cervical cancer), and U2OS (human bone osteosarcoma) cells were either mock-treated or IR treated with 10Gy and harvested 30 minutes after treatment. CDK2 was immunoprecipitated from cells using anti-CDK2 antibody and western blots were probed with anti-MRE11 antibody to detect the interaction. In control MEFs, the MRE11-CDK2 interaction was disrupted after IR, as seen in previous experiments.

However, in both cancer cell lines, the MRE11-CDK2 remained unaltered after induction of DNA DSBs. This data supports the notion that cancer cells, like *Atm*$^{-/-}$ cells, may be unable to initiate S-phase checkpoint pathways after DNA damage and that disruption of the MRE11-CDK2 interaction is needed for that to occur.

Discussion

In this study, we sought to further understand how the MRE11-CDK2 interaction functions in the DNA damage response and to decipher the biological functions of the MRE11 C-terminus. We have previously described how the MRE11-CDK2 interaction has roles in homologous recombination in normally dividing cells[32]. Our most recent research reveals that the MRE11-CDK2 interaction, though intact in normal cells, is disrupted after DSB induction in an ATM-dependent manner. While ATM is the master kinase of the DNA damage response, other DNA repair kinases could also be involved in regulating this interaction (i.e. ATR or DNA-PKcs), which warrants further investigation. Likewise, we only tested the interaction status after DSB induction caused by ionizing radiation. Future studies will need to investigate how other forms of damage, such as replication stress caused by the drug aphidicolin, effect the interaction between MRE11 and CDK2. Intriguingly, we did not observe disruption of the MRE11-CDK2 interaction after ionizing radiation treatment in human cancer cells, which leads us to speculate that the disruption after IR could be a mechanism to control CDK2 activity. It makes sense that this pathway would be altered in cancer cells – cells that bypass cell cycle checkpoints, accumulate DNA damage, and proliferate uncontrollably to form tumors. While we investigated this interaction in several human, mouse, and cancer cell lines, it remains to be seen if the MRE11-CDK2 interaction exists in cell lines we did not test.

Our lab has previously shown that the MRE11 C-terminus is both sufficient and required for interaction with CDK2[32]. In this recent study, we demonstrate

that the MRE11 C-terminus appears to regulate the disruption between MRE11 and CDK2 following IR treatment. However, the mechanism for IR-induced, ATM-dependent disruption of the MRE11-CDK2 interaction remains unclear. Previous studies demonstrated that the MRE11 C-terminus contains 5 ATM (and/or ATR) phosphorylation sites and that ATM phosphorylates these SQ/TQ sequences after IR[57, 58]. A phosphosite-mutant cell line in one of these reports exhibited decreased cell survival and increased chromosomal aberrations after radiation exposure, indicating these phoshosites have roles in DNA repair[59]. Here, we propose the mechanism for MRE11-CDK2 disruption after IR is ATM-dependent phosphorylation on one or many of the SQ/TQ sites on the MRE11 C-terminus. To test this hypothesis, we could mutate the Serines in the SQ/TQ sites to Alanines on the MRE11 C-terminus, making the protein non-phosphorylatable, and test its ability to interact with CDK2 before and after irradiation treatment. While this would be an exciting avenue to explore, our data counteracts this theory as we did not observe a band-shift (indicating phosphorylation) on a FLAG-tagged MRE11 C-terminal expressing construct treated with ionizing radiation. It is possible, however, that western blot analysis is not sensitive enough to detect this post-translational modification. To further investigate these questions, it would be interesting to utilize mass spectrometry, a more powerful technique, to examine post-translational modifications of the MRE11 C-terminus before and after DNA damage.

In this study, we demonstrate that ionizing radiation leads to disruption of the MRE11-CDK2 interaction, a reduction in catalytic CDK2 levels, and a decrease

in activating phosphorylation of CDK2 on Threonine 160. Next, we set out to determine how genetic disruption of the MRE11-CDK2 interaction effects CDK2 catalytic activity by examining basal levels of CDK2 kinase activity in MRE11-deficient and MRE11 C-terminal truncated cells compared to their matched controls. We observed lower levels in both MRE11-mutant genotypes. Additionally, we observed reduced levels of pCDK2 Threonine 160 in the MRE11-mutants. Together, this data suggests that genetic disruption of the MRE11-CDK2 interaction mimics IR-induced disruption of the interaction, both leading to reduced levels of CDK2 activity via decreased phosphorylation on CDK2 Threonine 160. We conclude that MRE11, specifically the C-terminus, has roles in controlling levels of CDK2 activity. Interestingly, MRE11-nuclease deficiency did not impact basal levels of CDK2 activity or activating CDK2 Threonine 160 phosphorylation, suggesting that MRE11's control over CDK2 activity in S-phase checkpoint functions is separate from its roles in DNA repair.

We hypothesize that disruption of the interaction is important for initiating an S-phase checkpoint pathway. Because the interaction is genetically disrupted in the MRE11 C-terminal truncation mutant, we predict that the S-phase checkpoint is always "on" on these cells. A constitutively active S-phase checkpoint would likely cause cells to proliferate slowly, independent of massive genomic instability. Indeed, we observed slow proliferation in cells lacking the MRE11 C-terminus, equivalent to the slow proliferation characteristic of MRE11-deficient MEFs[24]. Additionally, there was an increase in chromosome anomalies in the absence of the MRE11 C-terminus, but not to the same extent as those in cells lacking the

entire MRN complex. This data suggests that the mild genomic instability in the MRE11 C-terminal truncation mutant is insufficient to cause the slow proliferation phenotype and is instead due to low levels of CDK2 activity caused by genetic disruption of the MRE11-CDK2 interaction.

Next, we investigated how MRE11-deficiency and MRE11 C-terminal truncation impact responses to ionizing radiation during the S-phase checkpoint pathway. The S-phase checkpoint is an important anti-cancer barrier which detects DNA damage and replication stress and arrests cell cycle progression until the damage is repaired[33]. Previous studies have shown that normal cells inhibit CDK2 activity after IR as a mechanism for induction of the S-phase checkpoint[43, 44]. This process is defective in A-T patient cells, demonstrating that reduction in CDK2 activity after IR is ATM-dependent[43]. We found that complete loss of the MRN complex led to defects in inhibition of CDK2 activity after IR, while MRE11 C-terminal truncation still allowed for CDK2 inhibition in response to DNA damage. This data suggests that the MRN complex contributes to the inhibition of CDK2 activity after DNA damage, but not specifically through the MRE11 C-terminus. Inhibitory phosphorylation of CDK2 on Tyrosine 15 mirror these results as well.

The hallmark of the S-phase damage checkpoint is the slowing of replication in response to DNA damage. A-T patient cells exhibit radioresistant DNA synthesis, the inability to inhibit DNA replication in response to ionizing radiation[53]. Patient cells derived from individuals with ATLD and NBS have been found to exhibit an intermediate radioresistant DNA synthesis phenotype, while *Mre11*$^{ATLD1/ATLD1}$ mice, which express a patient allele causing truncation of the

MRE11 C-terminus, exhibited a severe radioresistant DNA synthesis phenotype[31, 44]. In our studies, MRE11-deficiency caused an intermediate radioresistant DNA synthesis phenotype, reminiscent of the studies in ATLD and NBS patients[44]. Loss of the MRE11 C-terminus, however, allowed for inhibition of DNA synthesis after IR. Patient and mouse ATLD cells have low levels of the MRN complex and loss of ATM-signaling, while our cell model for MRE11 C-terminal truncation has physiologic levels of the MRN complex and intact ATM signaling[30, 31]. Thus, we show that the S-phase checkpoint defects previously seen in *MRE11*^{ATLD1} human and *Mre11*$^{ATLD1/ATLD1}$ mouse cells must be due to low levels of the MRN complex, and not due to loss of the MRE11 C-terminus specifically. Intriguingly, this data supports our hypothesis of a constitutively active S-phase checkpoint in MRE11 C-terminally truncated cells, where we would not expect to observe S-phase checkpoint defects in response to IR.

Cell cycle checkpoint control is a universal mechanism in cells to prevent tumorigenesis[33]. The S-phase checkpoint is especially important because of its role in stalling replication to allow for damaged DNA to be repaired before cells divide in mitosis. Here we show that MRE11, a DNA repair protein, regulates CDK2's activity through its interaction with CDK2. Loss of the MRE11 C-terminus, which disrupts the interaction, results in lower levels of basal CDK2 activity, suggesting the C-terminus has specific roles in controlling CDK2 activity levels. Our proposed model is that disruption of the MRE11-CDK2 interaction after IR initiates an S-phase checkpoint pathway by reducing CDK2 catalytic activity. Therefore, our studies have revealed a novel S-phase checkpoint pathway

regulated by the MRE11 C-terminus. Future studies should further investigate the role of the MRE11-CDK2 interaction in cancer cells. If disruption of the interaction after DNA damage is important for initiating S-phase checkpoint function, one intriguing possibility is that disrupting the interaction synthetically in cancer cells using a small molecule inhibitor could restore S-phase checkpoint functions in these cells.

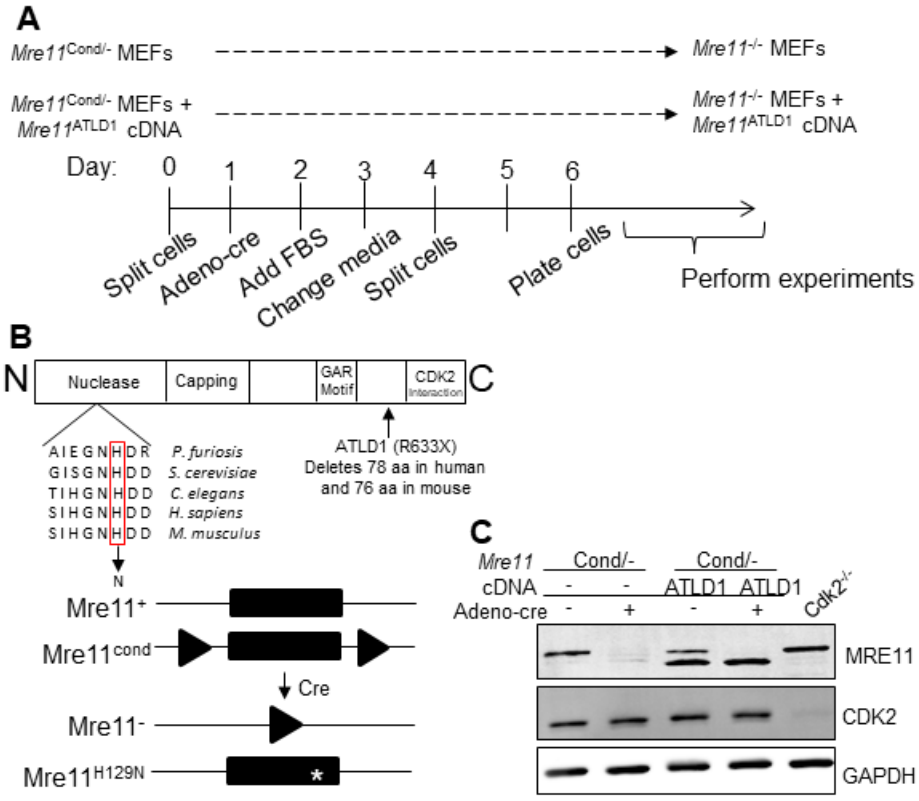
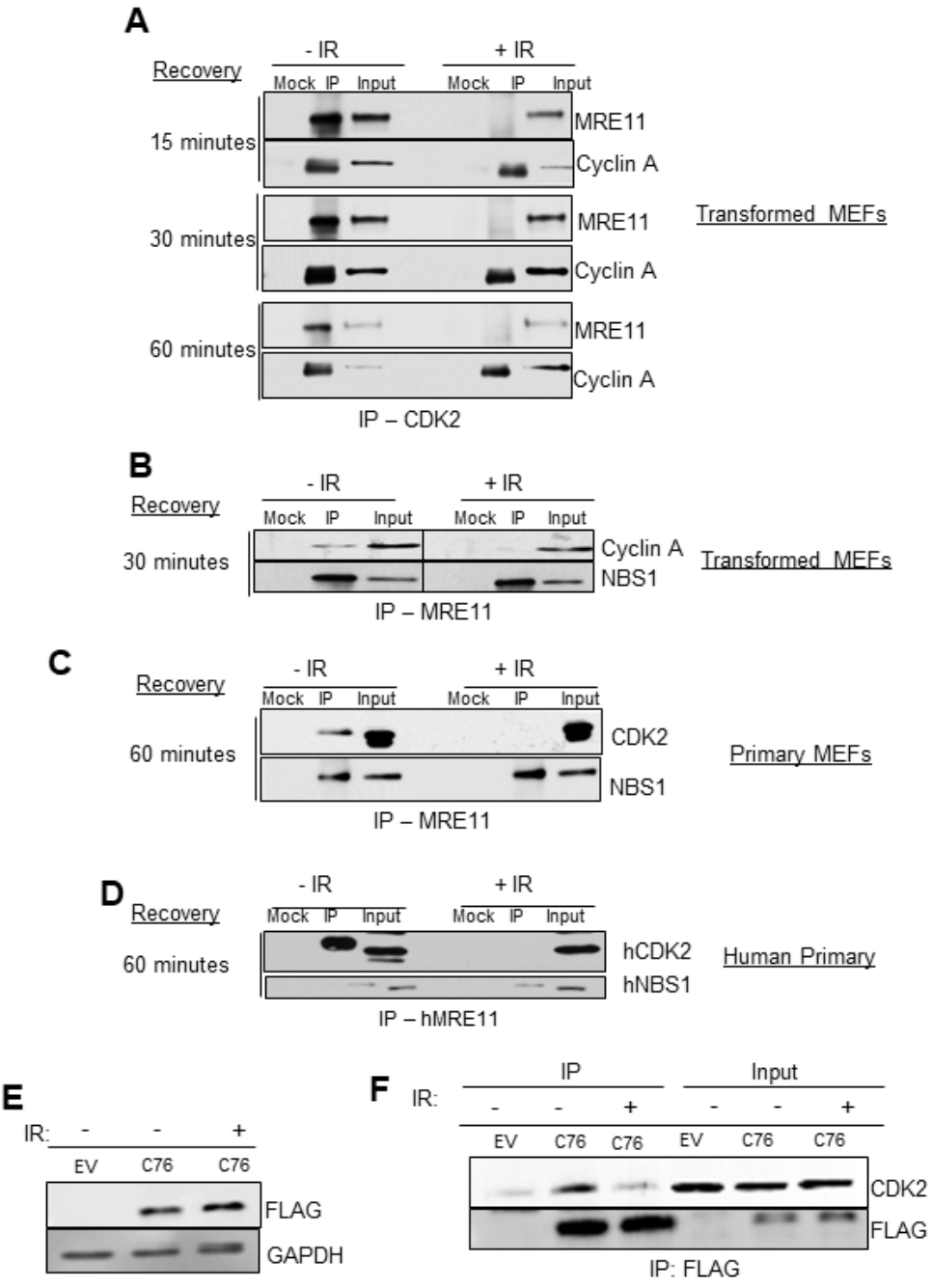


Figure 3.1. Experimental Approach. (A) *Mre11*$^{Cond/-}$ MEFs with or without the *Mre11*^{ATLD1} cDNA were treated with adeno-cre virus to delete endogenous MRE11 over the course of 5 days before plating for experiments. **(B)** Depiction of mammalian MRE11 domain structure and engineered murine germline *Mre11* alleles. The *MRE11*^{ATLD1} mutation causes C-terminal truncation of MRE11 by deleting the last 78 amino acids of the protein in humans and the last 76 amino acids of the protein in mice. **(C)** Western blot analysis showing MRE11 and CDK2 protein levels in MRE11-deficient and MRE11 C-terminal truncated expressing cells (*Mre11*$^{Cond/-}$ + *Mre11*^{ATLD1} cDNA).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Recovery
- IR
+ IR
Mock IP Input
Mock IP Input
15 minutes
30 minutes
60 minutes
MRE11
Cyclin A
Transformed MEFs
IP – CDK2
B
Recovery
30 minutes
Cyclin A
NBS1
Transformed MEFs
IP – MRE11
C
Recovery
60 minutes
CDK2
NBS1
Primary MEFs
IP – MRE11
D
Recovery
60 minutes
hCDK2
hNBS1
Human Primary
IP – hMRE11
E
IR: - - +
EV C76 C76
FLAG
GAPDH
F
IR:
IP
Input
- - + - - +
EV C76 C76 EV C76 C76
CDK2
FLAG
IP: FLAG

Figure 3.2. The MRE11-CDK2 interaction is disrupted after DNA damage induced by ionizing radiation. **(A)** CDK2 co-immunoprecipitations with MRE11 in transformed MEFs before and after a time-course treatment of ionizing radiation (IR). Western blot of co-IPs from *Mre11*$^{Cond/-}$ MEFs, using anti-CDK2 antibody (co-IP) or beads only (mock). 10Gy IR was used to induce DNA DSBs, and cells were harvested 15, 30, and 60 minutes after treatment. DNA damage disrupts the MRE11-CDK2 interaction at both early and late timepoints after IR treatment in transformed MEFs. **(B)** MRE11 co-immunoprecipitations with CDK2 in transformed MEFs before and after treatment with IR. Western blot of co-IPs from *Mre11*$^{Cond/-}$ MEFs, using anti-MRE11 antibody (co-IP) or beads only (mock). 10Gy IR was used to induced DNA DSBs and cells were harvested 30 minutes after treatment. DNA damage disrupts the MRE11-CDK2 interaction. **(C)** MRE11 co-immunoprecipitations with CDK2 in primary MEFs before and after treatment with IR. Western blot of co-IPs from primary MEFs, using anti-MRE11 antibody (co-IP) or beads only (mock). 10Gy ionizing radiation was used to induced DNA DSBs and cells were harvested 60 minutes after treatment. DNA damage disrupts the MRE11-CDK2 interaction in primary MEFs. **(D)** MRE11 co-immunoprecipitations with CDK2 in human primary cells before and after treatment with IR. Western blot of co-IPs from human primary cells, using anti-MRE11 antibody (co-IP) or beads only (mock). 10Gy IR was used to induced DNA DSBs, and cells were harvested 60 minutes after treatment. DNA damage disrupts the MRE11-CDK2 interaction in human primary cells. **(E)** Western blot analysis confirming transient transfection of 293T cells with either an empty vector or flag-tagged MRE11 C-terminus construct before and after IR. 10Gy IR was used to induce DNA DSBs, and cells were harvested 30 minutes after IR treatment. **(F)** Also shown are FLAG co-immunoprecipitations with CDK2 before and after treatment with IR. 293T cells were transfected with either an empty vector or a flag-tagged MRE11 C-terminus construct. 10Gy IR was used to induce DNA DSBs, and cells were harvested 30 minutes after treatment. Western blot analysis using anti-FLAG antibody (co-IP). The MRE11 C-terminus interacts with CDK2 under normal conditions, and treatment with IR disrupts that interaction.

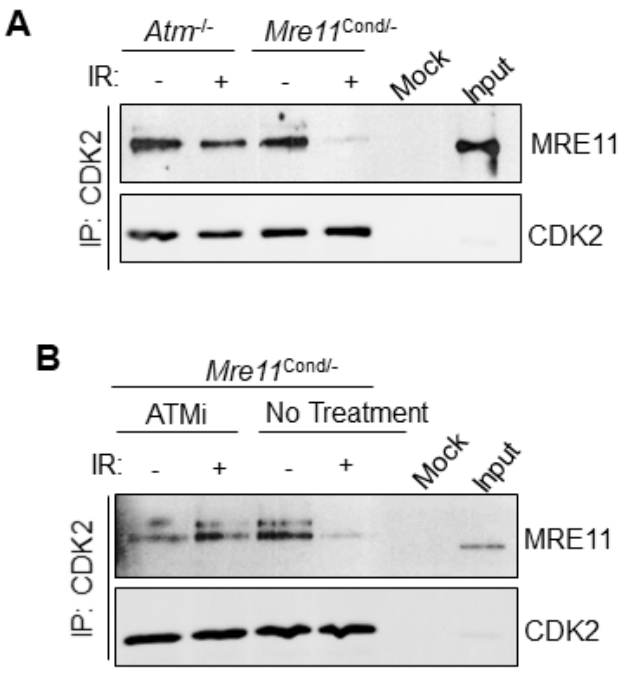
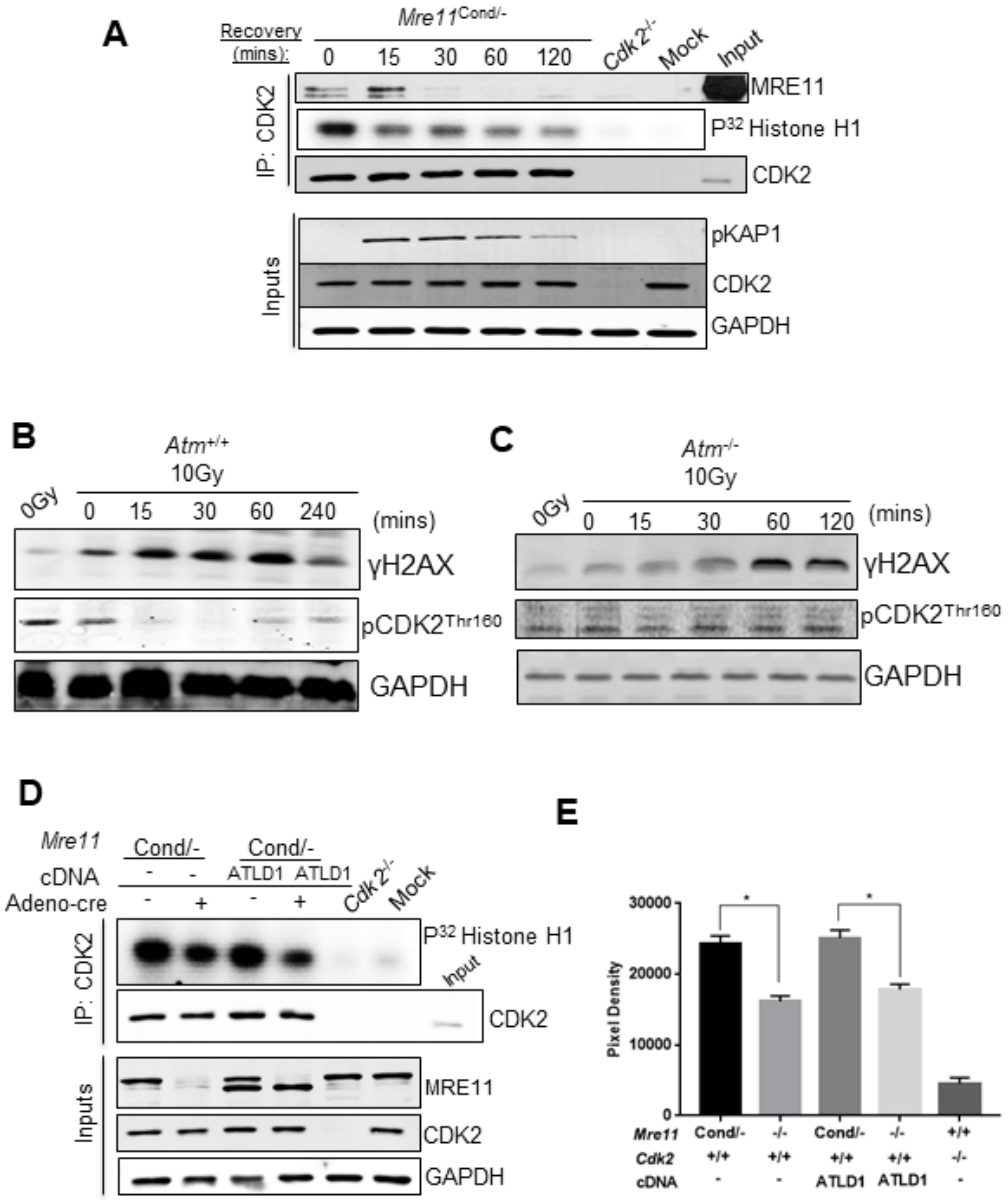


Figure 3.3. Disruption of the MRE11-CDK2 interaction after DNA damage is ATM-dependent. **(A)** CDK2 co-immunoprecipitations with MRE11 in transformed *Mre11*$^{Cond/-}$ MEFs and *Atm*$^{-/-}$ MEFs before and after treatment with IR. Western blot analysis using anti-CDK2 antibody (co-IP) or beads only (mock). 10Gy IR used to induce DNA DSBs, and cells were harvested 30 minutes after treatment. DNA damage disrupts the MRE11-CDK2 interaction in control cells after IR, but not in *Atm*$^{-/-}$ cells after IR. **(B)** CDK2 co-immunoprecipitations with MRE11 in transformed *Mre11*$^{Cond/-}$ MEFs before and after treatment with IR and an ATM inhibitor (10uM of the ATM inhibitor, KU55933, was used. Cells were pretreated for 1 hour with the inhibitor before exposure to IR). Western blot analysis using anti-CDK2 antibody (co-IP) or beads only (mock). 10Gy IR was used to induce DNA DSBs, and cells were harvested 30 minutes after treatment. DNA damage disrupts the MRE11-CDK2 interaction in untreated control cells after IR, but not in cells treated with an ATM inhibitor after IR.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Cdk2-/-
Mock
Input
IP: CDK2
MRE11
P32 Histone H1
CDK2
Inputs
pKAP1
CDK2
GAPDH
B
Atm+/+
10Gy
0Gy
0 15 30 60 240
(mins)
γH2AX
pCDK2Thr160
GAPDH
C
Atm-/-
10Gy
0Gy
0 15 30 60 120
(mins)
γH2AX
pCDK2Thr160
GAPDH
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Mre11
Cond/-
Cond/-
cDNA
- - ATLD1 ATLD1
Adeno-cre
- + - +
Cdk2-/-
Mock
IP: CDK2
P32 Histone H1
Input
CDK2
Inputs
MRE11
CDK2
GAPDH
E
Pixel Density
30000
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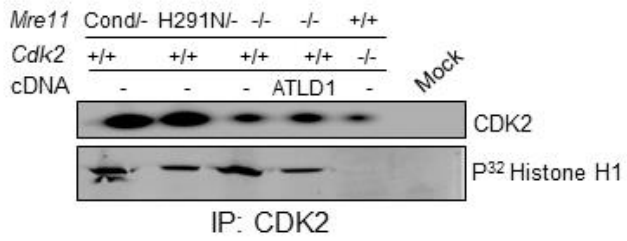
Cdk2 +/+ +/+ +/+ +/+ -/-
cDNA - - ATLD1 ATLD1 -

F


Mre11 Cond/- H291N/- -/- -/- +/+
Cdk2 +/+ +/+ +/+ +/+ -/-
cDNA - - - ATLD1 -
Mock
CDK2
P32 Histone H1
IP: CDK2

G

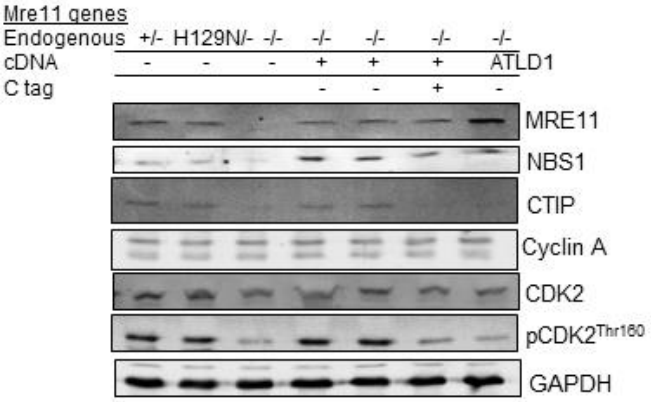
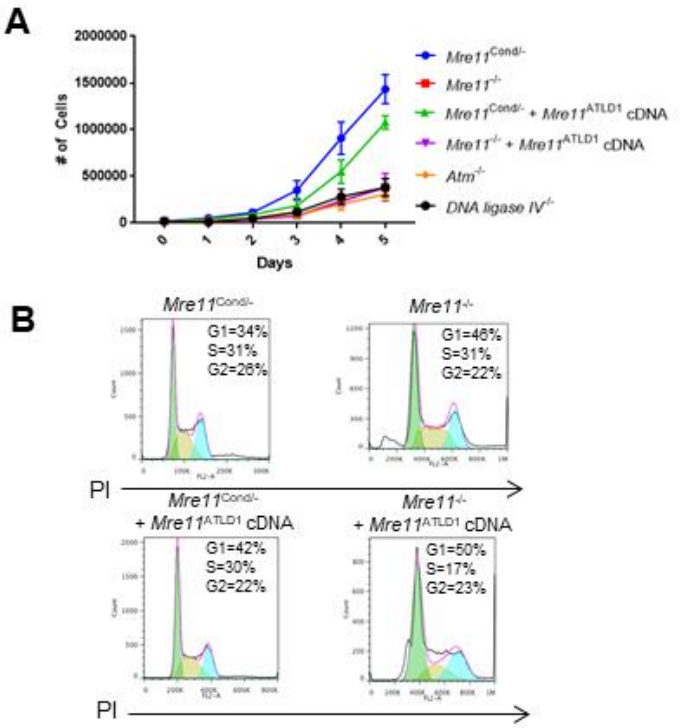
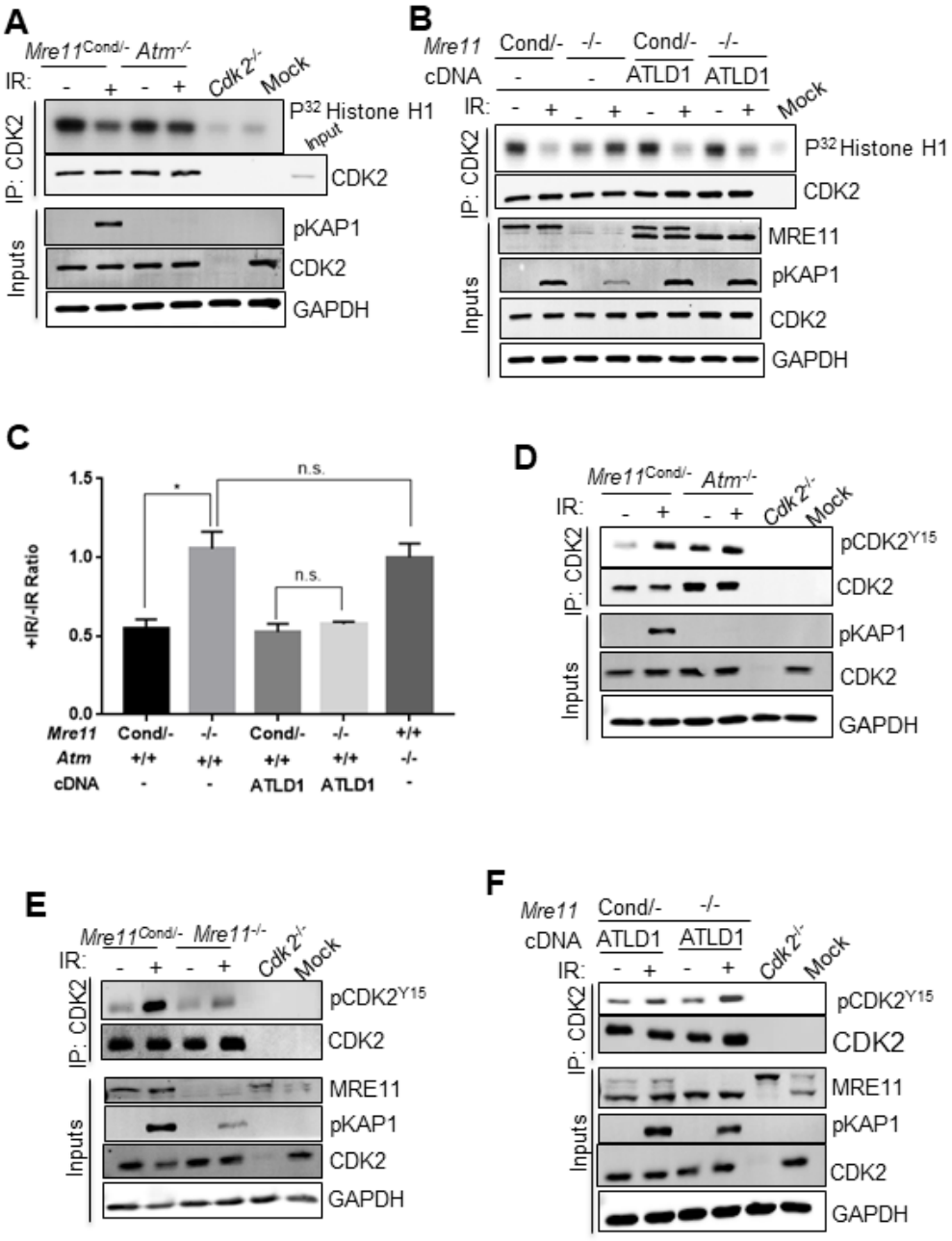

Mre11 genes
Endogenous +/- H129N/- -/- -/- -/- -/- -/-
cDNA - - - + + + ATLD1
C tag - - + -
MRE11
NBS1
CTIP
Cyclin A
CDK2
pCDK2Thr160
GAPDH

Figure 3.4. Ionizing radiation and genetically-induced disruption of the MRE11-CDK2 interaction cause reduction in phosphorylation of CDK2 on Threonine 160 and in CDK2 catalytic activity. (A) CDK2 kinase assay in transformed MEFS on a time-course of 15, 30, 60, and 120 minutes after 10Gy IR treatment. Also shown is CDK2 co-immunoprecipitation with MRE11, using anti-CDK2 antibody (co-IP) or beads only (no antibody). IR treatment causes a decrease in CDK2 activity levels and dissociation of the MRE11-CDK2 interaction. **(B)** Examination of pCDK2 Threonine 160 levels in *Atm*$^{+/+}$ cells on a time-course of 15, 30, 60, and 240 minutes after 10Gy IR treatment. DNA damage by IR caused pCDK2^{Thr160} levels to decrease at all timepoints, especially at 15 and 30 minutes following treatment. **(C)** Examination of pCDK2 Threonine 160 levels in *Atm*$^{-/-}$ cells on a time-course of 15, 30, 60, and 120 minutes after 10Gy IR treatment. DNA damage by IR did not cause pCDK2^{Thr160} levels to change following treatment. Therefore, inhibition of phosphorylation of CDK2 on Threonine 160 after IR is ATM-dependent. **(D)** CDK2 kinase assay in the following genotypes: *Mre11*$^{Cond/-}$, *Mre11*$^{-/-}$, *Mre11*$^{Cond/-}$ + *Mre11*^{ATLD1} cDNA, and *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing MEFs. *Cdk2*$^{-/-}$ MEFs were used as a negative control. Immunoprecipitations were performed with anti-CDK2 antibody or just beads (mock). MRE11-deficiency and Mre11 C-terminal truncation cause a reduction in basal levels of CDK2 catalytic activity. **(E)** Quantitation of 3 independent kinase assay experiments for the genotypes listed. CDK2 activity was quantified using Image J software and graphed using raw pixel density values. *, $P \leq 0.05$ (unpaired *t* test). **(F)** CDK2 kinase assay in the genotypes listed. Immunoprecipitations were performed with anti-CDK2 antibody or just beads (mock). MRE11-deficiency and MRE11 C-terminal truncation show a reduction in CDK2 catalytic activity. Loss of MRE11 nuclease activity did not cause a reduction in CDK2 activity. **(G)** Western blot analysis examining protein levels of MRE11, NBS1, CTIP, Cyclin A, pCDK2^{Thr160} and total CDK2 in indicated genotypes. GAPDH; loading control. Mre11$^{-/-}$ and *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing cells have lower basal pCDK2^{Thr160} levels compared to controls.



Spontaneous chromosomal anomalies					
Genotype	***Mre11*$^{Cond/-}$**	***Mre11*$^{-/-}$**	***Mre11*$^{Cond/-}$ + *Mre11*^{ATLD1} cDNA**	***Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA**	***Atm*$^{-/-}$**
Total Metaphases	163	141	151	153	164
Fragments	66 (0.40)	314 (2.23)	63 (0.42)	168 (1.10)	284 (1.73)
Breaks/Gaps	21 (0.13)	123 (0.87)	29 (0.19)	52 (0.34)	48 (0.29)
Dicentrics	2 (0.01)	53 (0.38)	10 (0.07)	17 (0.11)	15 (0.09)
Robertsonian Translocation	10 (0.06)	33 (0.23)	11 (0.07)	26 (0.17)	29 (0.18)
Radials	0 (0.00)	5 (0.04)	0 (0.00)	3 (0.02)	1 (0.01)
Circular Chromosomes	5 (0.03)	36 (0.25)	8 (0.05)	26 (0.17)	34 (0.21)
Total anomalies	104	564	121	292	411
Anomalies per metaphase	0.63	4.00	0.80	1.91	2.51

Figure 3.5. MRE11 C-terminal truncation results in slow proliferation equivalent to that of MRE11-deficiency, but only a mild genomic instability phenotype. (A) Proliferation curves in indicated genotypes over the course of 5 days in culture. *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing cells display a proliferation defect similar to that of *Mre11*$^{-/-}$ cells. **(B)** Cell cycle profiles of MRE11-mutant genotypes. A peak characterized by low DNA content was observed in MRE11-deficient cells, but not in the MRE11 C-terminal truncation mutant. This peak most likely signifies DNA fragmentation and loss of DNA content, a characteristic phenotype of cells with dramatic genomic instability. **(C)** Spectrum of chromosome anomalies in MRE11-mutant MEFs. Metaphase spreads were stained with DAPI and 4 independent experiments were conducted. The bar graph shows the number of anomalies per metaphase with types of anomalies indicated in the color key. The table shows proportions of anomalies per each genotype. C-terminal truncation of MRE11 results in genomic instability, but not to the same extent as MRE11-deficiency.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Mre11Cond/-
Atm-/-
IR:
Cdk2-/-
Mock
IP: CDK2
P32 Histone H1
Input
CDK2
pKAP1
Inputs
CDK2
GAPDH
B
Mre11
Cond/-
-/-
Cond/-
-/-
cDNA
ATLD1
ATLD1
IR:
Mock
IP: CDK2
P32 Histone H1
CDK2
MRE11
Inputs
pKAP1
CDK2
GAPDH
C
+IR/-IR Ratio
1.5
1.0
0.5
0.0
*
n.s.
n.s.
Mre11
Cond/-
-/-
Cond/-
-/-
+/+
Atm
+/+
+/+
+/+
+/+
-/-
cDNA
-
-
ATLD1
ATLD1
-
D
Mre11Cond/-
Atm-/-
IR:
Cdk2-/-
Mock
IP: CDK2
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CDK2
Inputs
pKAP1
CDK2
GAPDH
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Inputs
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F
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-/-
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ATLD1
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CDK2
Inputs
MRE11
pKAP1
CDK2
GAPDH

Figure 3.6. Examination of inhibitory phosphorylation of CDK2 on Tyrosine 15 and inhibition of CDK2 kinase activity after ionizing radiation treatment in MRE11-mutants. (A) CDK2 kinase assay in transformed *Mre11*$^{Cond/-}$ and *Atm*$^{-/-}$ MEFs before and after treatment with ionizing radiation. *Cdk2*$^{-/-}$ MEFs were used as a negative control. Immunoprecipitations were performed with anti-CDK2 antibody or just beads (mock). 10Gy IR treatment was used to induce DNA DSBs, and cells were harvested 30 minutes after treatment. *Atm*$^{-/-}$ cells are unable to inhibit CDK2 kinase activity after IR treatment. **(B)** CDK2 kinase assay in transformed *Mre11*$^{Cond/-}$, *Mre11*$^{-/-}$, *Mre11*$^{Cond/-}$ + *Mre11*^{ATLD1} cDNA, and *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing MEFs before and after treatment with IR. Immunoprecipitations were performed with anti-CDK2 antibody or just beads (mock). 10Gy IR treatment was used to induce DNA damage, and the cells were harvested 30 minutes after treatment. *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing cells inhibit CDK2 kinase activity after ionizing radiation treatment similar to controls, whereas *Mre11*$^{-/-}$ MEFS do not. **(C)** Quantitation of 3 independent kinase assay experiments for the genotypes listed. CDK2 activity was quantified using Image J software, and graphed as +IR/-IR ratio. *, $P \leq 0.05$ (unpaired *t* test). **(D)** CDK2 co-immunoprecipitations with pCDK2^{Y15} in transformed *Mre11*$^{Cond/-}$ and *Atm*$^{-/-}$ MEFs (*Cdk2*$^{-/-}$ MEFs were used as a negative control) before and after treatment with IR. Western blot analysis using anti-CDK2 antibody (co-IP) or just beads (mock). 10Gy ionizing radiation was used to induce DNA damage, and cells were harvested 30 minutes after treatment. pCDK2^{Y15} levels increase in control cells but not in ATM-null cells. **(E)** CDK2 co-immunoprecipitations with pCDK2^{Y15} in transformed *Mre11*$^{Cond/-}$ and *Mre11*$^{-/-}$ MEFs (*Cdk2*$^{-/-}$ MEFs were used as a negative control) before and after treatment with IR. Western blot analysis using anti-CDK2 antibody (co-IP) or just beads (mock). 10Gy IR was used to induce DNA damage, and cells were harvested 30 minutes after treatment. pCDK2^{Y15} levels do not increase in MRE11-null cells after ionizing radiation to the same extent as matched controls. **(F)** CDK2 co-immunoprecipitations with pCDK2^{Y15} in transformed *Mre11*$^{Cond/-}$ + *Mre11*^{ATLD1} cDNA and *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing MEFs (*Cdk2*$^{-/-}$ MEFs were used as a negative control) before and after treatment with IR. Western blot analysis using anti-CDK2 antibody (co-IP) or just beads (mock). 10Gy IR was used to induce DNA damage, and cells were harvested 30 minutes after treatment. pCDK2^{Y15} levels increase after ionizing radiation in *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing cells similarly to controls.

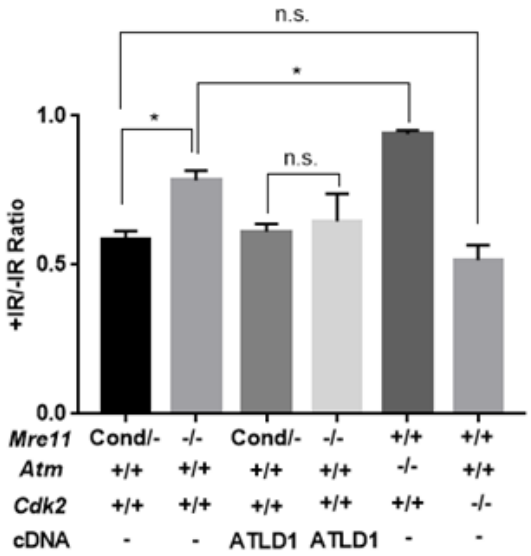


Figure 3.7. MRE11-deficient cells display an intermediate radio-resistant DNA synthesis phenotype while MRE11 C-terminal truncated expressing cells inhibit DNA synthesis after ionizing radiation treatment similar to controls. Radio-resistant DNA synthesis assay in genotypes listed. Cells were treated with 10Gy IR and harvested for the assay 4 hours post-treatment. A scintillation counter was used to obtain radioactivity levels and the data was graphed using a +IR/-IR ratio. At least three independent experiments were performed per genotype. *Atm*$^{-/-}$ MEFs displayed a classic radio-resistant DNA synthesis phenotype, whereas *Mre11*$^{-/-}$ MEFs were intermediate, and *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing cells looked similar to controls. *, $P \leq 0.05$ (unpaired *t* test).

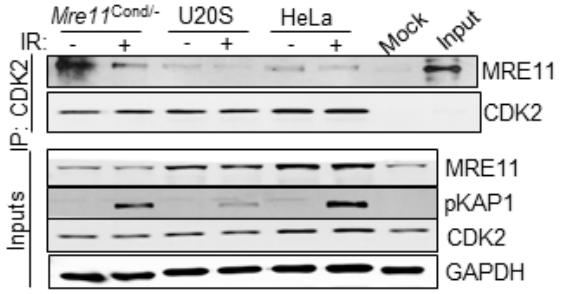


Figure 3.8. The MRE11-CDK2 interaction is not disrupted after DNA damage in the human U2OS and HeLa cancer cells lines. CDK2 co-immunoprecipitations with MRE11 in transformed *Mre11*$^{Cond/-}$ MEFs, U2OS cells, and HeLa cells before and after treatment with IR. Western blot of Co-IPs using anti-CDK2 antibody (co-IP) or beads only (mock). 10Gy IR was used to induce DNA DSBs, and cells were harvested 30 minutes after treatment. DNA damage disrupts the MRE11-CDK2 interaction in control MEF cells, but not in U2OS and HeLa cancer cells.

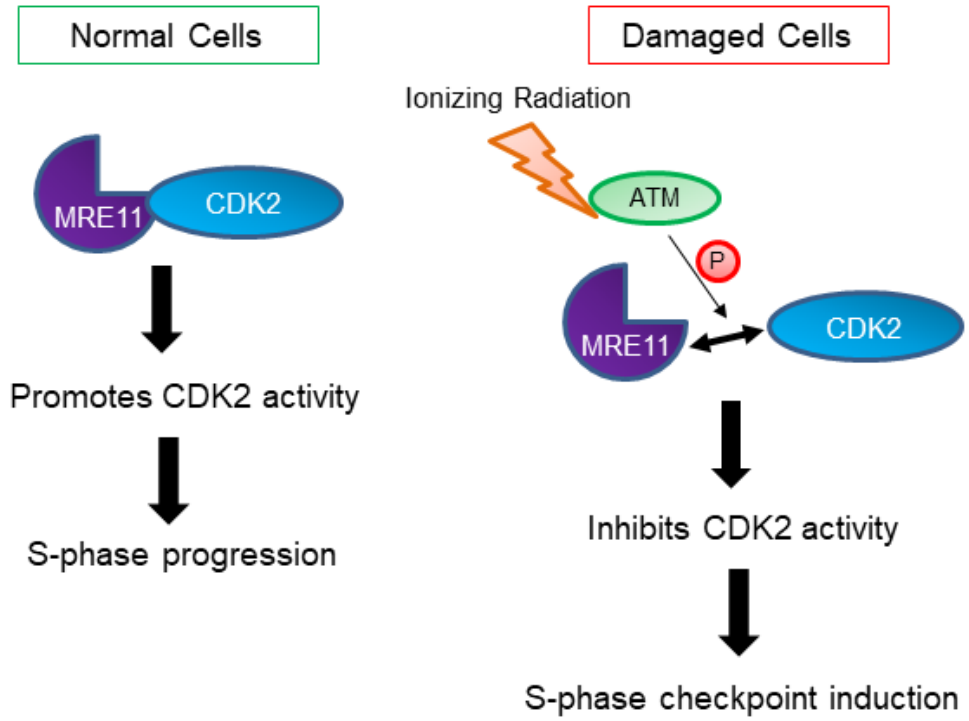
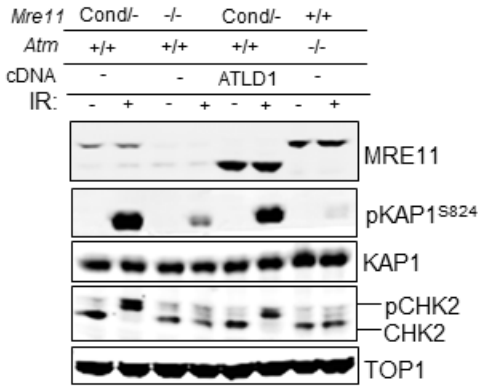
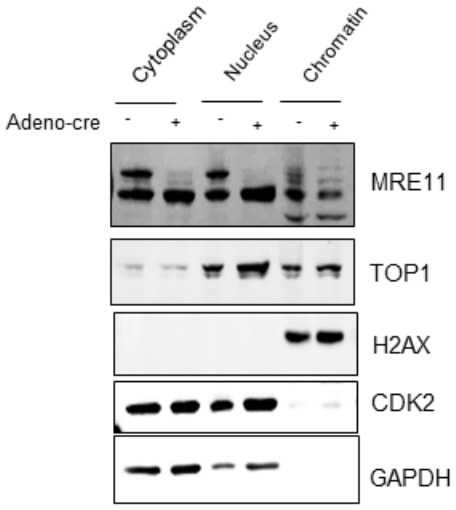


Figure 3.9. Model for MRE11-CDK2 interaction in the DNA double-strand break response. In normal cells, the MRE11-CDK2 interaction is intact, which promotes CDK2 activity and S-phase progression. Upon DSB induction by ionizing radiation, the MRE11-CDK2 interaction is disrupted in an ATM-dependent manner, leading to inhibition of CDK2 activity and S-phase checkpoint induction. Genetic disruption of the MRE11-CDK2 interaction mimics IR-induced disruption of the interaction, also leading to a reduction in CDK2 catalytic activity and a constitutively active S-phase checkpoint. Thus, MRE11, specifically the C-terminus, is necessary for maintaining normal levels of CDK2 activity.

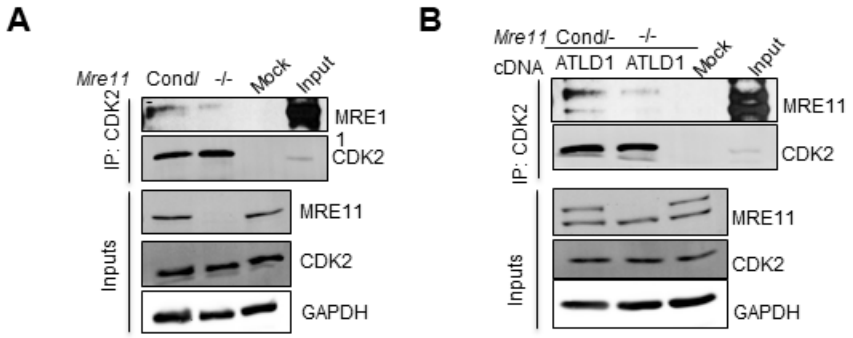


Supplemental Figure 3.1. ATM-signaling is defective in MRE11-deficient cells, but not in MRE11 C-terminal truncation expressing mutant. Western blot analysis examining phosphorylation of KAP1 and CHK2 (indicated by band-shift) in MRE11-mutant genotypes. Cells were treated with 10Gy IR and harvested 30 minutes post-treatment. TOP1, loading control.

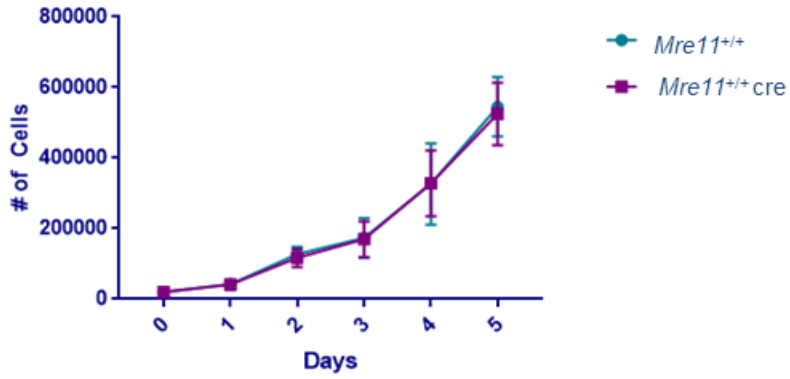


Supplemental Figure 3.2. MRE11 C-terminal truncated expressing cells localize to chromatin similarly to matched control cells. Fractionation experiments were performed to examine localization of MRE11 and CDK2 in the MRE11 C-terminal truncation mutant. Loss of the MRE11 C-terminus did not affect localization of MRE11 to the chromatin. Interestingly, we found that CDK2 does not localize at all to the chromatin, and is restricted to the cytoplasmic and nuclear fractions. H2AX was used as a marker for the chromatin.

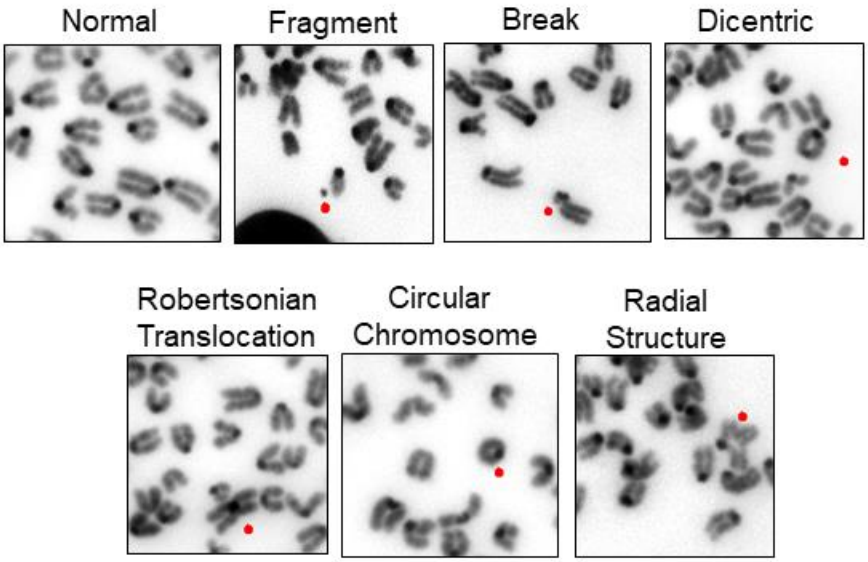
2



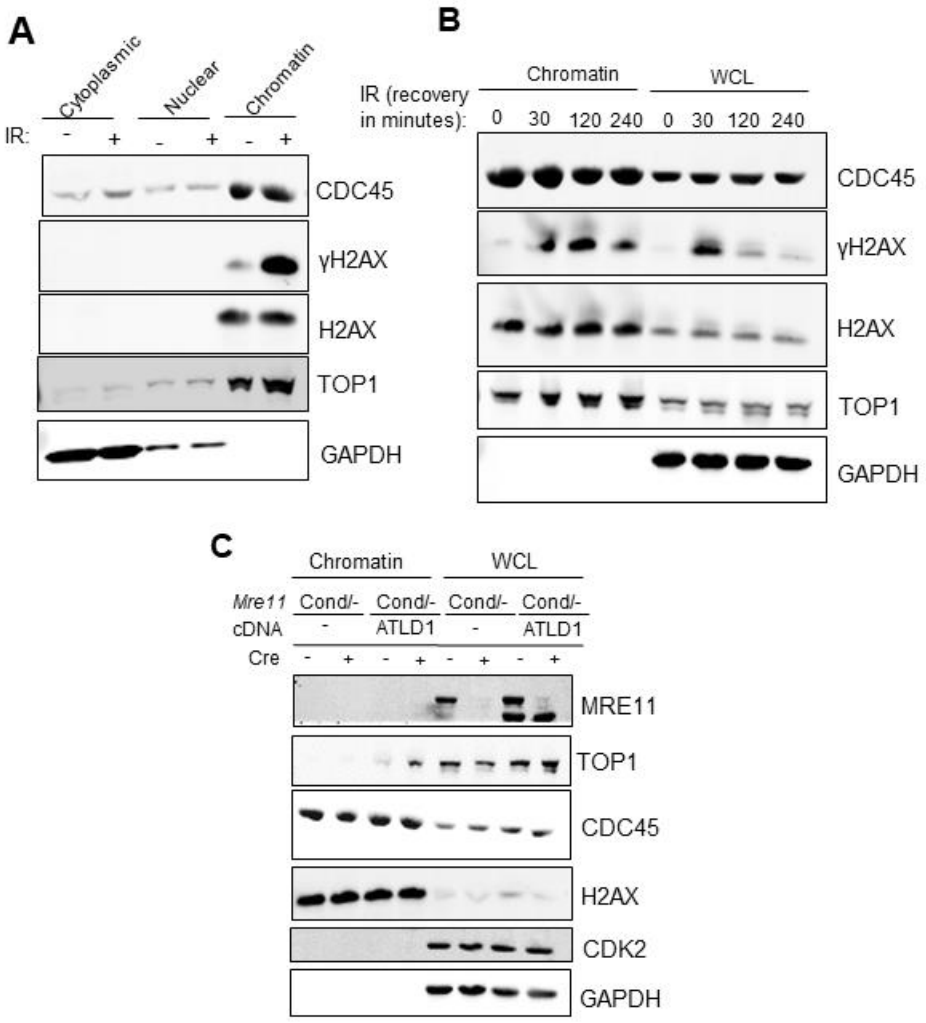
Supplemental Figure 3.3. MRE11-deficiency and MRE11 C-terminal truncation genetically disrupt the MRE11-CDK2 interaction. (A) CDK2 co-immunoprecipitations with MRE11 in transformed *Mre11*$^{Cond/-}$ and *Mre11*$^{-/-}$ MEFs. Western blot analysis using anti-CDK2 antibody (co-IP) or beads only (mock). The MRE11-CDK2 interaction is disrupted when MRE11 is absent. **(B)** CDK2 co-immunoprecipitations with MRE11 in transformed *Mre11*$^{Cond/-}$ + *Mre11*^{ATLD1} cDNA and *Mre11*$^{-/-}$ + *Mre11*^{ATLD1} cDNA expressing MEFs. Western blot analysis using anti-CDK2 antibody (co-IP) or beads only (mock). The MRE11-CDK2 interaction is disrupted when the MRE11 C-terminus is truncated.



Supplemental Figure 3.4. Cre-adenovirus treatment does not affect proliferation of *Mre11*$^{+/+}$ cells. To confirm that cre-adenovirus treatment did not have any off-target effects on cell growth, proliferation rates were measured in *Mre11*$^{+/+}$ cells and *Mre11*$^{+/+}$ cells treated with cre-adenovirus. Cells were plated and grown for 5 days in a 6 well dish, and counted each day using a hemocytometer and trypan blue exclusion. We did not observe any cell death upon addition of cre-adenovirus and treated cells grew similarly to non-treated controls.



Supplemental Figure 3.5. Representative images of each type of chromosomal anomaly examined in MRE11-mutant genotypes. Images show examples of normal chromosomes, fragments, breaks, dicentrics, Robertsonian translocations, circular chromosomes, and radial structures. Anomalies are indicated with a red dot. Images were taken using a 100x objective.


A
Cytoplasmic
Nuclear
Chromatin
IR:
- + - + - +
CDC45
γH2AX
H2AX
TOP1
GAPDH
B
IR (recovery in minutes):
Chromatin
WCL
0 30 120 240 0 30 120 240
CDC45
γH2AX
H2AX
TOP1
GAPDH
C
Chromatin
WCL
Mre11 Cond/- Cond/- Cond/- Cond/-
cDNA - ATLD1 - ATLD1
Cre - + - + - + - +
MRE11
TOP1
CDC45
H2AX
CDK2
GAPDH

Supplemental Figure 3.6. Examination of CDC45 chromatin loading in response to ionizing radiation and in the MRE11 C-terminal truncation mutant. **(A)** Biochemical fractionation was performed to analyze CDC45 chromatin loading in *Mre11*$^{Cond/-}$ cells before and after DNA damage induced by ionizing radiation. *Mre11*$^{Cond/-}$ cells were exposed to 10Gy radiation and fractionated one hour post-treatment. While we observed phosphorylation of H2AX after IR indicating induction of the DNA damage response, we did not observe the expected decrease in CDC45 loading after IR. **(B)** Biochemical fractionation in *Mre11*$^{Cond/-}$ cells treated with 10Gy ionizing radiation and harvested 0, 30, 120, and 240 minutes following treatment. We observed a minimal decrease of CDC45 loading at 120 minutes after IR, but not at earlier or later time-points. **(C)** Examination of CDC45 chromatin loading in the MRE11 C-terminal truncation mutant. Loss of the MRE11 C-terminus did not affect CDC45 levels on the chromatin compared to the matched control.

Materials and Methods

DNA constructs

The *Mre11*^{ATLD1} mutation was introduced into pEF6-MmMre11a using site directed mutagenesis (Stratagene). *Mre11*$^{Cond/-}$ MEFs were transfected (Lipofectamine 2000, Life Technologies) with the mutant MRE11-expressing construct and clones were then isolated and grown under blasticidin selection.

MEF engineering and cell culture

MEFs were derived in house as previously described[24]. Cells (MEFs, 293T, HeLa, and U20S) were grown in DMEM media (Invitrogen) supplemented with 10% FBS, 20mM HEPES (Corning), non-essential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen), 2mM L-glutamine (Invitrogen), and β-mercaptoethanol. For deletion of endogenous MRE11 protein, MEF cells were infected with Adeno-Cre retrovirus at an MOI of 500, grown for 5 days, split once, and then plated for experiments. Cells were exposed to a 137 Cesium source for ionizing radiation treatment when indicated.

Transient transfection of 293T cells

293T cells were plated onto 10 cm dishes and grown for 24 hours until 80-90% confluency. Cells were transfected with either empty vector or MRE11 C-terminal-Flag tagged cDNA, Lipofectamine 2000 (Thermo Fisher Scientific), and Optimem

(Thermo Fisher Scientific) according to the manufacturer's instructions. Transfections were confirmed by western blot analysis.

Western blot analysis

Cells were lysed in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50mM Tris, (pH 8.0)) or CSK buffer (50mM Tris-HCL, (pH 7.5), 150mM NaCl, 1% NP-40, and 10% Glycerol) containing protease and phosphatase inhibitors (Halt -Thermo Scientific Fisher) and spun down at high speed for 20 minutes, 4°C. Protein concentrations were determined using the Pierce Protein BCA Assay kit (Thermo Fisher Scientific). Denatured samples were resolved by SDS-PAGE and transferred to PVDF membranes (Immobilin).

Immunoprecipitation

Cells were harvested and lysed on ice in lysis buffer containing 50mM Tris- HCL (pH 7.5), 150mM NaCl, 1% NP-40, and 10% Glycerol supplemented with protease and phosphatase inhibitors (Halt – Thermo Fisher Scientific). Protein lysates were then incubated on ice for 30 minutes and spun down at 4°C for 20 minutes at high speed. Lysates were precleared with 10µL Protein A Agarose beads (Roche) for 20 minutes at 4°C on a rotator. Pierce BCA Protein Assay kit (Thermo Fisher Scientific) was used to calculate protein concentrations. 1.5 to 3 mg of protein were incubated with anti-CDK2 antibody or anti-Mre11 antibody overnight at 4°C on a rotating block. Approximately 18 hours later, 100µL of Protein A Agarose

beads (washed 3 times with lysis buffer) were added to the protein-antibody conjugates and allowed to mix for 1 hour at 4°C. Beads were then washed 3-5 times with lysis buffer. Immunoprecipitates were eluted with 2x Laemmli buffer (Bio-Rad) at 95°C for 10 minutes. Samples were resolved on SDS-PAGE and transferred under normal conditions.

Antibodies

Primary antibodies used in this study are as follows: Cyclin A (Santa Cruz), CDC45 (Santa Cruz), CDK2 (Cell Signaling), pCDK2^{Thr160} (Cell Signaling), pCDK2^{Y15} (Abcam), CHK2 (Cell Signaling), CTIP (Santa Cruz), FLAG (Santa Cruz), GAPDH (Santa Cruz), H2AX (Cell Signaling), γH2AX (Millipore), KAP1 (Cell Signaling), pKAP1 (Bethyl), MRE11 (Cell Signaling), NBS1 (Novus), and TOP1 (BD Pharmingen). Secondary antibodies for western blot analysis were: IRDye800CW-conjugated goat anti-rabbit/anti-mouse (Li-Cor Biosciences) or peroxidase goat anti-rabbit/anti-mouse (Jackson Immunolabs).

Kinase Assay

1x10^6 cells were plated onto 10cm dishes and allowed to grow for 48 hours. Cells were treated with 10Gy of ionizing radiation and allowed to recover for indicated timepoints post-irradiation. Next, cells were harvested and lysed on ice in lysis buffer containing 20mM Tris (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% (w/v) Triton X-100, 2.5mM sodium pyrophosphate, 1mM B-glycerophosphate,

1mM Na3VO4, and 1mM leupeptin. Protein extracts were spun down for 20 minutes at 4°C at high speed and protein concentrations were measured using the Pierce BCA kit (Thermo Fisher Scientific). Protein A Agarose beads (Roche) were washed 3 times in lysis buffer (5 minutes each). 1.5-3 mg of protein was loaded onto 100µL of washed beads, along with anti-CDK2 antibody (Cell Signaling), and incubated together overnight at 4°C while rotating. 18 hours later, the beads were washed 3 times in lysis buffer. Beads were then equilibrated and washed 3 times in kinase buffer containing 50mM Tris-HCL (pH 7.5), 150mM NaCl, 10mM $MgCl_2$ and 1mM DTT (dithiothreitol; stock is 1M in H_2O). Next, the kinase reactions were performed whereby the kinase reaction mix was added to the purified CDK complexes. Each reaction contains the purified CDK complex with antibody, protein A-agarose beads mixed with 2ug Histone H1 protein (Roche), 50uM "cold" ATP (Roche), 10µCi γ-^{32}P-ATP (Perkin Elmer), in a total of 25µL kinase buffer. The reactions were mixed well and were incubated for 45 minutes in a 37°C incubator. Samples were denatured in 2x Laemmli buffer (Bio-Rad) for 10 minutes at 95°C and resolved on acrylamide gels under normal conditions. Kinase activity was evaluated using autoradiography using film and/or a 9400 Typhoon Scanner (GE Healthcare). Steps using radioactivity were performed behind a β-shield to protect the experimenter from harmful radiation.

Metaphase analysis

$1x10^6$ cells per genotype were plated onto 10 cm dishes and allowed to grow for 48 hours. Colcemid (KaryoMAX) solution (20µL colcemid per mL of media) was

added to the cells for an incubation time of 4 hours at 37°C. The cells were then trypsinized, pelleted, and washed 2 times with PBS. 0.4% KCL solution was prewarmed at 37°C and added slowly to the cells with gentle agitation to disrupt any clumps and then incubated at 37°C for 15 minutes. Cells were then fixed with fresh ice-cold fixative (3:1 methanol: glacial acetic acid). After fixing, cells were dropped onto glass slides and dried on a slide warmer at 65°C. Nuclei were stained with DAPI (Invitrogen). Metaphases were viewed on an Olympus microscope under 100X objective and analyzed with SKYview Software (Applied Spectral Imaging).

Radioresistant DNA Synthesis Assay

Cells were seeded onto 6 well dishes at a density of $1x10^5$ cells/well (unirradiated controls and irradiated samples in duplicate) and cultured for 48 hours in thymidine-free media (RPMI1640). After 48 hours, cells were irradiated or mock-irradiated at a dose of 10Gy. The media was immediately changed to tritiated HEPES media (methyl-3H Thymidine at 2.0uCi/mmol [Perkin Elmer]), along with unlabeled thymidine to final molar concentration of 1µM, and the cells were allowed to recover for 4 hours. Next, the media was removed, and the cells were washed with warm PBS. The cells were then incubated at 37°C in unlabeled media for an additional 20 minutes to chase the labeled precursor pool. After 20 minutes, the media was removed, and the cells were again washed with PBS. 500µL of fresh 0.25M NaOH was used to lyse the cells which were mixed with a pipette and transferred to scintillation vials filled with 7.5mL of scintillation cocktail suitable for

alkaline solutions (Hionic Fluor from Packard). Samples were immediately taken to a scintillation counter for counting and analysis.

Proliferation Assay

Cells were plated in 6 well dishes at a density of $2x10^4$ cells/well and counted every day for 5 days using a hemocytometer and trypan blue exclusion.

Cell cycle profiles

Cells were harvested, fixed with 70% ethanol, and stained with 50µg/mL propidium iodide (PI), 50µg/mL RNase A, 0.1% Triton-X solution for 20 minutes and then analyzed by FACs analysis on an Accuri C6 flow cytometer. FlowJo software was used to analyze cell cycle profiles.

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Chapter IV

MRE11 Promotes Tumorigenesis by Facilitating Resistance to Oncogene-Induced Replication Stress‡

Abstract

Hypomorphic mutations in the genes encoding the *MRE11/RAD50/NBS1 (MRN)* complex lead to human syndromes associated with cancer. MRN is a sensor of DNA double-strand breaks (DSBs) and is required for recruitment and activation of the ataxia-telangiectasia mutated (ATM) kinase, which triggers DNA repair pathways and cell-cycle checkpoints. To gain a deeper understanding of the roles of the MRN complex in cancer, our lab engineered mice with B-lymphocytes lacking either the entire MRN complex or lacking the nuclease activities of MRE11. Both forms of MRN deficiency led to characteristics of cancer, including oncogenic translocations between C-MYC and the immunoglobulin locus

‡ Contents of this chapter have been published in the following paper and have been modified for this thesis:

Elizabeth Spehalski*, Kayla M. Capper*, Cheryl J. Smith*, **Mary J. Morgan**, Maria Dinkelmann, Jeffrey Buis, JoAnn Sekiguchi, and David O. Ferguson. MRE11 Promotes Tumorigenesis by Facilitating Resistance to Oncogene-Induced Replication Stress. *Cancer Research,* (2017). *****Equal Contribution

MJM contributed to the mouse studies in Figure 4.1
ES contributed to Figures 4.1 and 4.3A
KMC contributed to Figures 4.3B and 4.4-5
CJS contributed to Figure 4.2

(Ig). Interestingly, these preneoplastic B lymphocytes did not progress to detectable B-cell lineage lymphoma, even in the absence of the tumor suppressor, p53. In addition, MRE11 deficiencies prevented tumorigenesis in a mouse model strongly predisposed to spontaneous B-cell lymphomas. These findings indicate that MRN is not a classic tumor suppressor and instead imply that the nuclease activities of MRE11 are required for oncogenesis. Inhibition of MRE11 nuclease activity increased DNA damage and selectively induced apoptosis in cells overexpressing the C-MYC and N-MYC oncogenes, suggesting that MRE11 serves an important role in countering oncogene-induced replication stress. Together, these data imply that MRE11, specifically its nuclease activities, could serve as a therapeutic target in cancers overexpressing the oncogene MYC.

Introduction

Inherited syndromes that cause cancer-predisposition often arise from mutations in genes involved in the cell cycle checkpoints and DNA repair. DNA DSBs are highly toxic lesions and failure of cellular responses to DSBs cause diseases with diverse pathological symptoms, including cancer pre-disposition[1]. One example is Li-Fraumeni syndrome, caused by germline mutations in the *TP53* gene, which plays important roles in cell cycle regulation upon DNA damage. These patients present with clinical phenotypes such as sarcomas, leukemias, adrenal cortical carcinomas, and other cancers[2]. Similarly, inherited deficiencies in the *ATM* gene cause the disease ataxia-telangiectasia (A-T), which also leads to lymphomas and other cancers in patients[3].

ATM, a serine/threonine kinase, is activated in response to DSBs and initiates signaling cascades that elicits DNA repair mechanisms, cell-cycle checkpoints, and apoptosis. The recruitment and activation of ATM primarily depends on the MRN complex[4, 5]. MRE11's nuclease activities initiate end-resection during repair by homologous recombination (HR)[6-8]. The functional relationship between MRN and ATM is highlighted by the fact that mutations in the *MRN* complex cause inherited genetic syndromes with phenotypes like those found in A-T patients. Partial loss-of-function mutations in *MRE11* cause the disease ataxia-telangiectasia-like disorder (ATLD), which is characterized by cerebellar ataxia and variable predisposition to cancer[9, 10]. Hypomorphic mutations in the *NBS1* gene lead to Nijmegen breakage syndrome (NBS) and mutations in *RAD50* lead to NBS-like disorder. Patients with NBS exhibit severe

predisposition to cancer, immunodeficiency, mental retardation, and microcephaly[11, 12].

The cancer-prone phenotypes associated with MRN syndromes suggest the complex functions as a tumor suppressor. Two Japanese brothers with ATLD reportedly died of pulmonary adenocarcinoma at ages 9 and 14 and were shown to have two distinct *MRE11*-mutant alleles[10]. Furthermore, mouse models harboring NBS or ATLD disease alleles are commonly prone to cancer, which reinforces the notion that MRN acts as a tumor suppressor[13-15]. However, complete knockout of any of the components of the *MRN* complex results in early embryonic lethality in mice[16-18]. This is likely the same for humans as no known disease alleles are null. Thus, human disease alleles must preserve some functions of the MRN complex necessary for mammalian development.

Our lab has previously generated a germline mouse allele in which *Mre11* can be conditionally deleted, leading to deficiency of the entire MRN complex[16]. In addition, we have targeted a single amino acid change in the endogenous *Mre11* locus that expresses a mutant MRE11 ($Mre11^{H129N}$) deficient in DNA nuclease activities, while preserving the integrity of the MRN complex[16]. Studies of mice and cells with combinations of these alleles have demonstrated that the nuclease activity of MRE11 is essential for embryonic development and for DSB repair[16, 19]. However, the nuclease activity of MRE11 is not required for ATM activation, thus providing a clear separation of function between involvement in DNA repair and ATM-dependent damage and cell cycle checkpoint responses.

In this study, we utilized our engineered *Mre11* mouse alleles in combination with *p53* deficiency to further understand roles for MRN in cancer. Mice were generated in which mature B lymphocytes were either deficient for the entire MRN complex, or MRE11 nuclease activity, which preserves functions of the rest of the complex. Surprisingly, MRE11 deficiency and MRE11 nuclease deficiency did not predispose mice to detectable B-cell malignancy. Furthermore, MRE11 deficiency prevented the formation of tumors in a genetic background highly prone to early B-cell lymphomas. From this data, we conclude that MRN is not a classic tumor suppressor. It appears, instead, that certain functions of the complex, such as MRE11 DNA nuclease activity, may be required for oncogenesis.

Results

Establishing MRE11 deficiencies in the murine B-lymphocyte lineage

The programmed DSBs and rearrangements that exist in B-lymphocytes makes this cell lineage ideal to study roles of aberrant DSB repair in cancer initiation and progression. These mistakes in DSB repair are directly responsible for oncogenic translocations between the immunoglobulin heavy chain locus (IgH) and proto-oncogenes, such as C-MYC[20]. In mice, B lineage tumors harbor IgH:MYC translocations. Therefore, we generated mice that lack MRE11 entirely, or express an MRE11 nuclease-deficient allele, in the B-lymphocyte lineage to evaluate the impact on the initiation or progression of lymphomagenesis[16, 19].

In order to overcome the embryonic lethality conferred by absence of MRE11 (*Mre11*$^{-/-}$) or defective MRE11 nuclease activity (*Mre11*$^{H129N/-}$), we utilized a conditional allele (Mre11^{cond}) that can be inactivated through cre-mediated recombination of two LoxP sites flanking a conserved exon within the endogenous *Mre11* locus. Through breeding, we generated *Mre11*$^{+/cond}$, *Mre11*$^{-/cond}$, and *Mre11*$^{H129N/cond}$ mice, each containing one allele of CD19-cre, which expresses cre recombinase in bone marrow B-lymphocyte progenitors[16, 19]. Cre expression can be detected in pro-B cells in bone marrow, prior to class switch recombination (CSR), which takes place in mature B-cells in peripheral lymphoid organs[21]. This breeding scheme resulted in mice with B cells of the following genotypes: *Mre11*$^{+/-}$, *Mre11*$^{-/-}$, and *Mre11*$^{H129N/-}$ (Figure 4.1A).

Examination of survival and tumorigenesis in mice with deficiencies in MRE11 and P53

Elizabeth Spehalski and I aged mice with B-cell genotypes *Mre11*$^{+/-}$ (control), *Mre11*$^{-/-}$, and *Mre11*$^{H129N/-}$, with and without *p53*, to evaluate survival and tumor development. p53 wild-type mice with B-cell-specific MRE11 deficiencies were found to have a normal life span with a median survival of 27.5 months (compared with 29 months for the controls) (Figure 4.1B). Mice with *p53*$^{-/-}$*Mre11*$^{-/-}$ and *p53*$^{-/-}$*Mre11*$^{H129N/-}$ B-cell genotypes died at about 16 to 18 weeks of age (Figure 4.1C). Upon necropsy, the majority of *p53*$^{-/-}$ mice presented with an enlarged thymus that was negative for the B-cell marker B220 (data not shown). Additional flow cytometry analyses of these tumors indicated that the masses were thymic lymphomas of T-cell origin, which is a common outcome of murine p53 deficiency[22, 23]. A smaller percentage of mice had non-lymphoid tumors. This data suggests that B lymphocyte-specific deficiency of MRE11, or MRE11 nuclease activity, does not significantly impact the survival of mice. Furthermore, these studies imply that there is no predisposition to B-cell malignancy in these mice despite the presence of genome instability and defective p53-dependent cell-cycle checkpoints (Figure 4.1D).

MRE11 B-cell specific mutation suppresses pro-B lymphoma in an *Artemis/p53* double-null background

Next, to further assess the roles of MRE11 in tumorigenesis, we examined the impact of MRE11 deficiencies in a mouse model that is strongly predisposed to B-cell lineage lymphomas[24]. Cheryl Jacobs Smith performed these experiments. The ARTEMIS DNA nuclease is required for processing of DNA ends during V(D)J recombination, a lymphocyte-specific DNA rearrangement that generates antibody diversity[20]. V(D)J recombination is required for lymphocyte development and mutations in *ARTEMIS* cause immunodeficiency disorders and in some patients, cancer predisposition[25]. Previous studies have demonstrated that *Artemis*-null mice, when combined with p53 mutation, succumb to lymphoid tumors that display clonal translocations involving IgH and *C-Myc* or *N-Myc* genomic loci[24]. In this study, we used the *Artemis*$^{-/-}$*p53*$^{-/-}$ mouse model of spontaneous pro-B lymphomagenesis to determine the impact of MRE11 deficiency or MRE11 nuclease deficiency on tumor development.

We introduced the target *Mre11* mutant alleles into the *Artermis/p53* double-null background through a complex mouse breeding scheme and obtained *Artemis*$^{-/-}$*p53*$^{-/-}$*Mre11*$^{-/cond}$ and *Artemis*$^{-/-}$*p53*$^{-/-}$*Mre11*$^{H129N/cond}$ mice harboring the CD19-cre transgene. We observed that the *Artemis*$^{-/-}$*p53*$^{-/-}$ mice expressing wild-type MRE11 in B cells had a median survival of 14 weeks (Figure 4.2A). In comparison, mice with B-cell genotypes of *Artemis*$^{-/-}$*P53*$^{-/-}$*Mre11*$^{-/-}$ and *Artemis*$^{-/-}$*p53*$^{-/-}$*Mre11*$^{H129N/-}$ exhibited median survival times of 17 and 19 weeks, respectively

(Figure 4.2A). These studies show that B-cell-specific loss of MRE11 or MRE11 nuclease activity did not markedly alter survival of these tumor-prone mice.

The majority of *Artemis*$^{-/-}$*p53*$^{-/-}$ mice analyzed succumbed to progenitor B-lymphoma, and smaller subsets had thymic lymphoma of progenitor T-cell origin or other tumor types, which are consistent with previous studies[24, 26]. Analyses of the pro-B tumors confirmed that they had clonal IgH rearrangements and amplifications of the IgH and *C-Myc* or *N-Myc* loci (data not shown). We observed that nearly all of the mice analyzed harboring B-lineage MRE11 deficiencies also succumbed to tumors. One *Artemis*$^{-/-}$*p53*$^{-/-}$*Mre11*$^{H129N/-}$ mouse became moribund and died with no obvious cause of death upon analysis. Surprisingly, no pro-B-cell lymphomas arose in *Artemis*$^{-/-}$*p53*$^{-/-}$ mice with either the *Mre11*$^{-/-}$ (n=11) or *Mre11*$^{-/H129N}$ (n=11) B cell genotypes. The mice in these cohorts primarily developed thymic lymphomas, and in one *Artemis*$^{-/-}$*p53*$^{-/-}$*Mre11*$^{-/H129N}$ case, a pre-B cell tumor. Importantly, this tumor as well as all of the other lymphoid tumors analyzed retained the *Mre11* conditional allele and expressed MRE11 protein. These results indicate that MRE11 mutation suppresses pro-B-cell lymphomas in tumor-prone mice where they normally arise (Figure 4.2B).

Detection of translocations in MRE11-deficient cells

We next investigated the broader roles of the MRN complex in generating chromosomal translocations by examining sites outside of the rearranging IgH locus. Because the MRN complex has been implicated in all known DSB repair pathways, it is possible that translocations cannot be generated in the absence of

MRE11. To answer this important question, Elizabeth Spehalski stably integrated a plasmid (DR-GFP) harboring an 18 base pair target site for the rare cutting endonuclease I-SceI in *Mre11*$^{-/cond}$ MEFs. The conditional *Mre11* allele was deleted by the addition of cre adenovirus to generate *Mre11*$^{-/-}$ cells[16]. Mre11^{H129N} cDNA was also stably expressed in cells to analyze the impact of MRE11 nuclease deficiency in translocation formation. I-SceI can cleave the mouse genome at cryptic sites that diverge 1-5 nucelotides from the consensus sequence[27]. Erroneous repair between the I-SceI induced DSB in the integrated substrate and cryptic sites results in translocation between the two loci, which can be detected by PCR amplification. After introduction of I-SceI via adenovirus, we detected translocations between the stably integrated I-SceI substrate and the endogenous *Mmp24* gene on chromosome 2, which contains a cryptic cut site in control cells[27]. PCR products were cloned and sequenced to confirm the presence of translocations (data not shown). In *Mre11*$^{-/-}$ MEFS and *Mre11*$^{-/-}$ MEFs expressing the Mre11^{H129N} cDNA, we also detected PCR products that were confirmed to be translocations (Figure 4.3A). This data demonstrates that translocations can be generated in fibroblasts that lack either MRE11 entirely or lack MRE11 nuclease activity.

We next examined the spontaneous chromosomal anomalies that arise in *Mre11*$^{-/-}$ and *Artemis*$^{-/-}$*Mre11*$^{-/-}$ MEFs for the presence of fusions and translocations by analyzing metaphase spreads. This experiment was performed by Kayla Capper. Upon adeno-cre deletion of MRE11, we observed a marked increase in chromosomal anomalies in *Mre11*$^{-/-}$ MEFs in comparison with control cells,

consistent with previous studies[16]. We found that combined mutation of ARTEMIS and MRE11 resulted in a moderate increase in chromosomal anomalies as compared to MRE11 deficiency alone. It is worthy to note that MRE11 deficiency alone or in combination with ARTEMIS deficiency displayed elevated levels of chromosome and chromatid fusions, including dicentrics, ring chromosomes, and Robertsonian translocations as compared to controls. These observations provide evidence that chromosomal translocations can be generated in the absence of MRE11 in nonlymphoid cell types at loci outside of the rearranging IgH locus, and also in cells lacking the ARTEMIS DSB repair nuclease.

DNA damage responses in MYC oncogene overexpressing cells

Many activated oncogenes drive inappropriate entry into and progression through S-phase of the cell cycle, which can lead to replication stress in the form of collapsed replication forks that result in the generation of DSB intermediates[28]. The presence of DSBs activates DNA damage responses that can be detected in some tumors[28]. The DNA damage response reflects an attempt to maintain genome stability, but has the unintended consequence of preserving the viability of cancerous cells. We therefore examined the DNA damage responses triggered by overexpression of the oncogene MYC.

To induce MYC function in the nucleus, Kayla Capper generated human U2OS cell lines stably expressing the human MYC cDNA fused to the hormone-binding domain of the estrogen receptor (MYC-ER)[29]. Upon 4-hydroxytamoxifen (4-OHT) treatment, MYC translocates to the nucleus where it becomes active and

leads to increased accumulation of cells in the S-phase (data not shown)[30]. We examined the localization of DNA repair proteins to sites of damage, which can be detected as punctate subnuclear foci by immunofluorescence microscopy. Key DNA repair proteins, including phosphorylated H2AX (γH2AX) p53-binding protein 1 (53BP1), single strand DNA-binding protein (RPA), Fanconi anemia group D2 protein (FANCD2), and breast cancer 1, early onset (BRCA1), localize to stalled replication forks and facilitate their restart and/or repair. We observed that MYC overexpression induced increased foci formation of γH2AX (among others) compared with controls (Figure 4.4A). Importantly, we also observed increased localization of the NBS1 component of MRN to sites of damage in response to MYC oncogene overexpression (Figure 4.4B). These findings indicate that MYC overexpression induces genomic damage that triggers recruitment of the MRN complex to DNA DSBs.

MRE11 deletion or inhibition in oncogene-overexpressing cells reduces cellular survival

Findings not shown here indicate that loss of MRE11 results in decreased proliferation in cells overexpressing MYC. To selectively examine the importance of MRE11 nuclease activity in MYC overexpressing cells, Kayla Capper utilized Mirin, a well-characterized small molecular inhibitor of MRE11. Mirin was first identified in 2008 in a forward genetic screen of 10,000 compounds[31]. Studies have shown that Mirin specifically inhibits MRE11 exonuclease activity, while maintaining the integrity of MRN complex formation and ATM signaling[31]. First,

the impact of inhibition of MRE11 nuclease activity by Mirin on survival of the human U2OS cell line overexpressing MYC was assessed. Upon 4-OHT treatment, exposure to Mirin resulted in markedly reduced survival at concentrations of the inhibitor that had minimal impact on survival of control cells (Figure 4.5A). To determine the cause of reduced viability of cells overexpressing MYC, the extent of apoptosis in the cells induced by Mirin was examined. We observed that the percentage of cells undergoing apoptosis as measured by Annexin V staining was significantly increased in Mirin-treated cells overexpressing MYC in a dose-dependent manner. In contrast, at similar doses of the MRE11 nuclease inhibitor, control cells were mostly unaffected (Figure 4.5B). These results indicated that the nuclease activity of MRE11 is important for survival of cells overexpressing oncogenes, and a small molecular inhibitor of MRE11 nuclease activity can induce selective killing of MYC-expressing cells.

Discussion

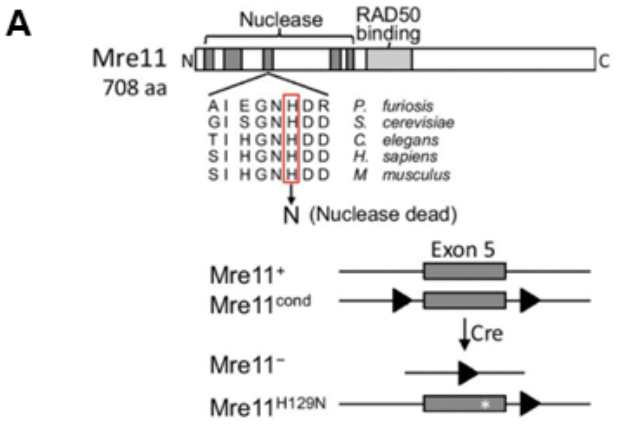
In this study, we sought to further understand roles of the MRN complex in cancer development. To this end, we generated mice with MRE11 deficiency or MRE11 nuclease deficiency in the B-lymphocyte lineage. Splenic B cells displayed hallmarks of cancer, such as genome instability and the formation of oncogenic IgH:Myc translocations. Despite these observations, and the defective ATM-dependent checkpoint responses inherent to loss of MRN, there was a striking absence of B-lymphocyte malignancy, even in a p53 null background. This data implies that partial loss of the *MRN* complex in inherited patient alleles retains functions of the complex necessary for tumor formation. The nuclease activities of MRE11 appear to be one such function. In support of this notion, we found that loss of the MRN complex or MRE11 nuclease activity prevents pro-B lymphomagenesis in the *Artemis*$^{-/-}$*p53*$^{-/-}$ tumor-prone mouse model.

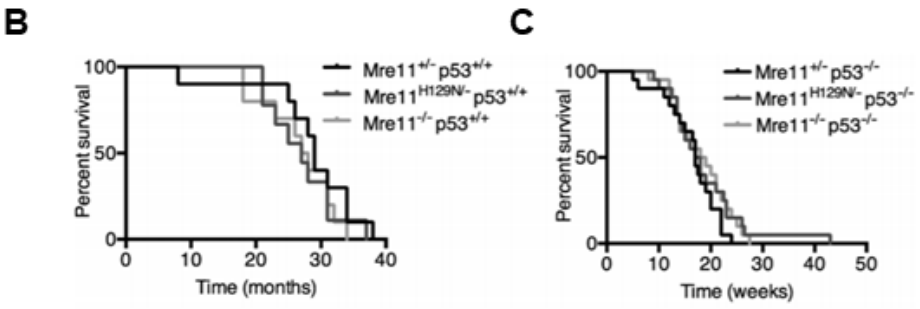
The lack of tumors was even more surprising when compared with deficiencies of other factors involved in DSB repair. When XRCC4 was absent in mature mouse B-lymphocytes, persistent chromosome breaks in IgH were observed at about the same frequency we observed with MRE11 deficiency. In this case, however, mature B-lineage lymphomas did form when XRCC4 deficiency was combined with germline p53 deficiency[32]. Furthermore, mouse models harboring targeted germline null mutations in *DNA ligase IV*, *Xrcc4*, or *Artemis*, combined with *p53* deficiency, were predominately predisposed to pro-B-cell lymphomas characterized by translocation and co-amplification of the *IgH* and *Myc* loci (and IgH:N-MYC) translocations in the case of ARTEMIS deficiency)[24,

33, 34]. This is in striking contrast to deficiency of the MRN complex or MRE11 nuclease activities, where we found a noticeable absence of these tumors. We cannot entirely exclude the possibility that the p53-deficient mice succumb to thymic lymphomas before B-cell lymphomas can be detected. However, tissues from *Mre11/p53*-mutant mice at ages beyond development of tumors in the *Xrcc4/p53*-deficient mice showed no sign of malignancy.

One hypothesis for the absence of B-cell tumors in MRE11 deficiency is that oncogenes, like MYC, play a role in tumor development in conjunction with MRE11. Previous studies have shown that overexpression of MYC deregulate S-phase, which increases replication stress and generates significant genomic instability[28]. Secondly, MRE11 and its nuclease activities are required for cellular survival in response to replication stress[16, 35, 36]. Thus, it is possible that overexpression of oncogenes coupled with defective cellular responses to replication stress is incompatible with the cellular proliferation and survival required for tumor growth[37]. To this end, we demonstrate that cells overexpressing oncogenes exhibit enhanced DNA damage responses, decreased survival, and increased apoptosis upon inactivation of MRE11 nuclease activity. Together, these findings suggest that although inherited hypomorphic mutations of *MRN* components lead to cancer predisposition, complete loss of *MRN* may not be compatible with malignancy. Thus, the hypomorphic mutations of *MRN* must retain specific functions of the complex, likely MRE11 nuclease activity, that support cancer development.

Our studies raise the possibility that pharmacologic inhibition of MRE11 nuclease activity could represent a promising new avenue in cancer therapeutics (Figure 4.6). Consistent with this notion, we demonstrate that inhibition of MRE11 nuclease activity by the small-molecule inhibitor, Mirin, selectively decreases survival of oncogene expressing human cells. Specifically targeting the nuclease activities of MRE11 would disable the ability of tumor cells to endure oncogene-induced replication stress, while maintaining responses controlled by the ATM kinase. This approach warrants further study, especially in light of the development of the next generation of selective MRE11 nuclease inhibitors[6].



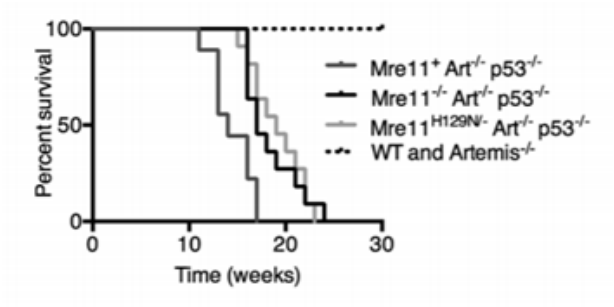


D

	B-cell genotype		
Cause of death	**Mre11$^{+/-}$p53$^{-/-}$**	**Mre11$^{H129N/-}$p53$^{-/-}$**	**Mre11$^{-/-}$p53$^{-/-}$**
B-cell lymphoma	0 (0%)	0 (0%)	0 (0%)
Thymic lymphoma	14 (70%)	13 (65%)	15 (75%)
Non-lymph tumor	2 (10%)	3 (15%)	1 (5%)
Non-tumor	4 (20%)	4 (20%)	4 (20%)
n	20	20	20

Figure 4.1. Examination of survival and tumorigenesis in mice with deficiencies in MRE11 and P53. **(A)** Mammalian MRE11 domains and location of the invariant histidine required for nuclease activity (top). The four murine germline *Mre11* alleles used in this study (bottom). **(B)** Kaplan-Meier plots showing the survival percentages of mice with B-cell genotypes of $Mre11^{+/-}$, $Mre11^{H129N/-}$, and $Mre11^{-/-}$ in a p53 wild-type background. The median survival of $Mre11^{-/-}p53^{+/+}$ mice was 27.5 months while the median survival of mice with $Mre11^{H129N/-}p53^{+/+}$ mice was 29 months. **(C)** Kaplan-Meier plots showing the survival percentages of mice with B-cell genotypes of $Mre11^{+/-}$, $Mre11^{H129N/-}$, and $Mre11^{-/-}$ in a p53-null background. The median survival of $Mre11^{-/-}p53^{-/-}$ mice was 17.5 months while the median survival of mice with $Mre11^{H129N/-}p53^{-/-}$ mice was 18.5 months. **(D)** Table depicting causes of death for mice with the indicated B-cell genotypes. No mice developed B-cell lymphomas.

A



B

	B-cell genotype		
Cause of death	**Mre11^{+} Art$^{-/-}$ p53$^{-/-}$**	**Mre11$^{H129N/-}$ Art$^{-/-}$ p53$^{-/-}$**	**Mre11$^{-/-}$ Art$^{-/-}$ p53$^{-/-}$**
ProB Lymphoma	12 (63%)	0 (0%)	0 (0%)
TCRβ- T-cell lymphoma	6 (32%)	9 (82%)	11 (100%)
Pre-B lymphoma	1 (5%)	1 (9%)	0 (0%)
Non tumor	0 (0%)	1 (9%)	0 (0%)
n	19	11	11

Figure 4.2. MRE11 B-cell specific mutation suppresses pro-B lymphoma in an *Artemis*/*p53* double-null background. (A) Kaplan-Meier survival curve showing the percent survival of mice versus age in weeks. Mice were observed for survival and tumor development for 30 weeks. *Artemis*$^{-/-}$*p53*$^{-/-}$ *Mre11*$^{cond/-}$ and *Artemis*$^{-/-}$*p53*$^{-/-}$*Mre11*$^{Cond/H129N}$ mice harboring the CD19-cre transgene survive longer than *Mre11*$^{+}$*Artemis*$^{-/-}$*p53*$^{-/-}$ mice. **(B)** Table depicting the causes of death and tumor types arising in *Artemis/p53/Mre11*-mutant mice. No pro-B lymphomas were observed in *Artemis*$^{-/-}$*p53*$^{-/-}$ mice with germline *Mre11*$^{Cond/-}$ or *Mre11*$^{Cond/H129N}$ alleles and CD19-cre.

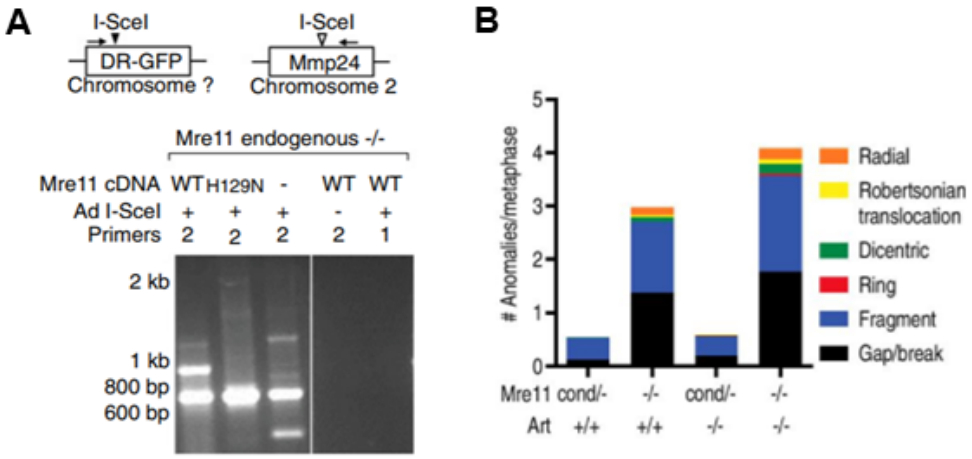


Figure 4.3. Detection of translocations in MRE11-deficient cells. (A) The top panel is a schematic of PCR-based detection of I-SceI endonuclease-induced translocations. Nested PCR primers detect translocations between the randomly integrated I-SceI site (black arrowhead) and endogenous, cryptic I-SceI site within the *Mmp24* locus (open arrowhead) on Chromosome 2. The bottom panel shows ethidium bromide agarose gels of nested PCR products from cells expressing $Mre11^{WT}$, $Mre11^{H129N}$, or no $Mre11^{-}$. Addition of Ad I-SceI and nested (2) or first round only (1) PCR primers. **(B)** Spontaneous chromosomal anomalies in $Mre11^{-/-}$ and $Mre11^{-/-}Art^{-/-}$ MEFs. Metaphases were stained with DAPI and scored in a blinded manner for chromosomal anomalies. The bar graph shows the number of anomalies per metaphase and the key shows the type of anomaly as indicated in by each color.

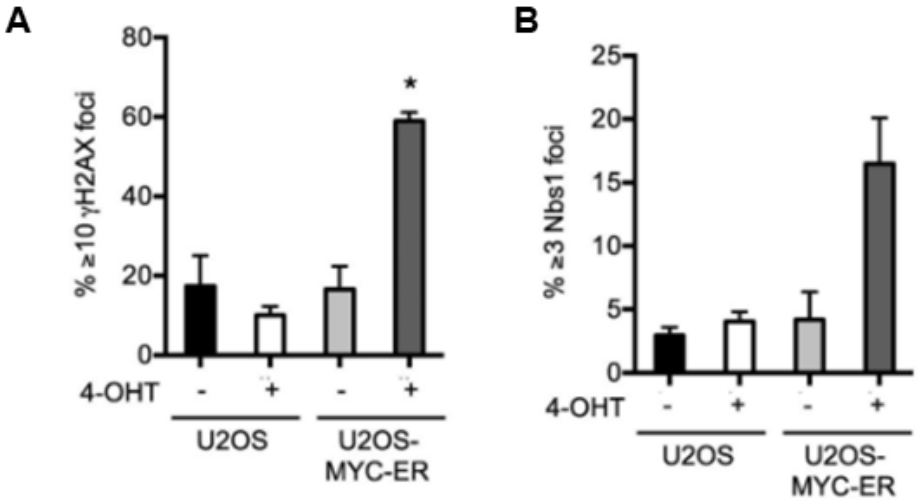


Figure 4.4. DNA damage responses in MYC oncogene overexpressing cells. Examination of DNA repair foci upon MYC overexpression in human U2OS cells. U2OS-pBABE and U2OS-MYC-ER cells were treated with DMSO or 200nmol/L 4-OHT to induce MYC translocation to the nucleus. Localization of DNA repair proteins at sites of DNA damage was detected by immunofluorescence microscopy. Percentage of nuclei with **(A)** γH2AX and **(B)** NBS1 foci is plotted. Mean ± SEM for three independent experiments is graphed. *, $P \leq 0.05$; paired *t* test.

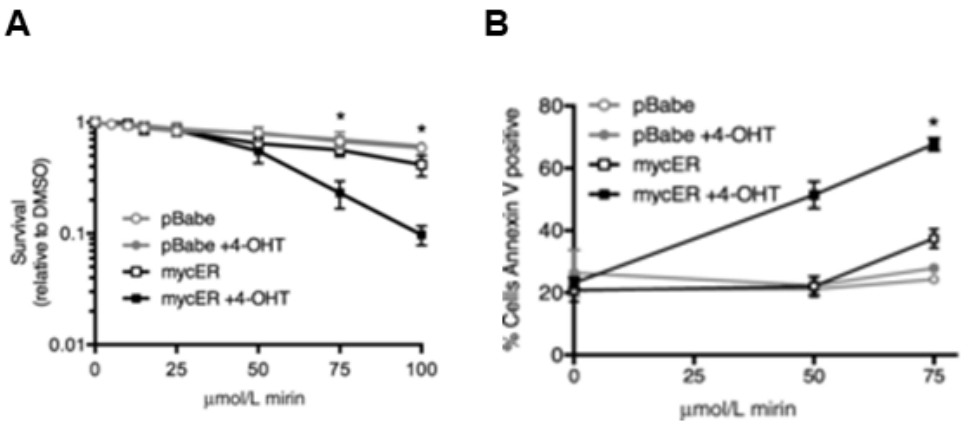


Figure 4.5. MRE11 deletion or inhibition in oncogene-overexpressing cells reduces cellular survival. (A) MYC overexpressing human U2OS cells exhibited decreased survival upon treatment with the small molecule inhibitor, Mirin. U2OS-pBABE and U2OS-MYC-ER cells were treated with DMSO or 4-OHT for 72 hours, then with increasing concentrations of Mirin for 24 hours. Cellular survival after 48 hours was determined relative to vehicle controls. Graph represents mean ± SEM of three independent experiments. *, $P \leq 0.05$. **(B)** Cells were treated as in **(A),** and the percentages of Annexin V-positive cells were determined by flow cytometry.

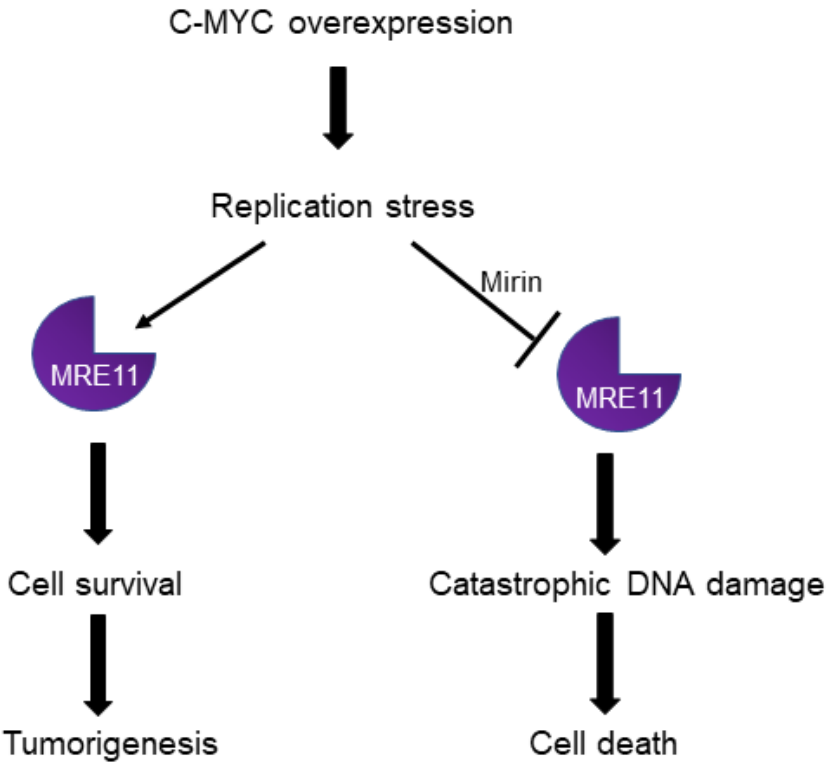


Figure 4.6. Model for targeting MRE11 nuclease activity in C-MYC oncogene-induced replication stress. C-MYC overexpression causes replication stress, a phenomenon that prevents error-free replication and stalling of DNA synthesis in cancer cells. In some cancer cells, replication stress may be countered by the DNA damage response, which allows for their survival and promotes tumorigenesis, but also results in the accumulation of DNA damage. We show that further enhancing replication stress, by inhibiting the nuclease activity of MRE11 with the small molecule inhibitor, Mirin, leads to catastrophic DNA damage and apoptosis in cancer cells.

Materials and Methods

Mouse Models

Mre11$^{cond/+}$, *Mre11*$^{cond/-}$, and *Mre11*$^{cond/H129N}$ mice with CD19-Cre expression were generated in our lab. P53 mice were obtained from The Jackson Laboratory. The gene targeted *Artemis*-null mice are as previously described[38]. Mice were housed in a pathogen-free facility.

Derivation and transformation of murine embryonic fibroblasts

Primary murine embryonic fibroblasts (MEF) of less than 4 passages were immortalized with SV40 large T antigen or MYC using pLenti-Myc T58A (generous gift from Dr. David Lombard, University of Michigan). Primary MEFs were derived in our laboratories and confirmed by PCR-based genotyping, sequencing, and Western blot analyses. Experiments were performed on cells ranging from 5 to 10 passages after immortalization.

Translocation PCR

DR-GFP:Mmp24 translocations were identified by a nested PCR reaction using Expand Long Template PCR System (Roche).

MRE11 inhibitor treatment of MYC-overexpressing cells

U2OS cells were originally obtained from the ATCC. U2OS-pBABE and U2OS-cMycER cell lines were generated by infecting the human U2OS cell line with the retroviral vector pBABE-PURO or pBabepuro-MYC-ER (Addgene plasmid #19128) expressing human c-MYC cDNA fused to the hormone-binding domain of the estrogen receptor (ER), then selected in puromycin (2.5 mg/mL). For cellular survival assays, U2OS-pBABE and U2OS-cMycER cells were plated in triplicate in 96-well plates 24 hours before treatment with 200 nmol/L 4-OHT (Sigma). After 72 hours, cells were treated with mirin (Cayman Chemical) or DMSO for 24 hours, and survival was determined by fixing and staining cells with Crystal Violet then measuring absorbance of solubilized dye at 595 nm. Percent survival was calculated relative to vehicle-treated controls. Three or more independent experiments were performed.

Apoptosis Assay

U2OS-pBABE and U2OS-cMycER cells were plated in 6-well plates 24 hours before treatment with 200 nmol/L 4-OHT (72 hours) and then treated with mirin for 24 hours. After 48 hours, cells were harvested and stained with FITC Annexin V antibody (BD Pharmingen 556419) and propidium iodide and then analyzed by flow cytometry (BD Accuri C6 Flow Cytometer). Data were analyzed using FlowJo software.

Metaphase spread analyses

Mre11$^{Cond/-}$ and *Artemis*$^{-/-}$*Mre11*$^{Cond/-}$ MEFs were infected with adeno-empty or adeno-cre viruses at an MOI of 500. Cells were grown for 3 days, split, doubly infected then incubated with colcemid (KaryoMAX) for 8 hours. DAPI-stained chromosomes were imaged on an Olympus BX61 microscope using 60× objective (SKYview software; Applied Spectral Imaging). Chromosomal anomalies were scored in a blinded manner from at least three independent experiments.

Immunofluorescence microscopy

U2OS-pBABE and U2OS-cMycER cells were plated on coverslips (4×10^4) in a 12-well dish (24 hours) and then treated with 200 nmol/L 4-OHT for 72 hours. Fixed cells were stained with primary antibodies (45 minutes), and then secondary antibodies, Alexa Fluor 488 or 594 (45 minutes). Foci were visualized using an Olympus BX61 microscope using 100× objective.

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Chapter V

Discussion

Roles for MRN in DNA damage kinase activation

Summary and Insights

In this thesis, I investigated roles for the MRN complex in DNA damage kinase activation, cell cycle checkpoint control, and tumorigenesis. In Chapter II, I sought to further understand the interplay between the two main double-strand break (DSB) sensors, the MRE11/RAD50/NBS1 (MRN) complex and the KU70/KU80 heterodimer, and activation of their respective kinases, ataxia-telangiectasia mutated (ATM) and DNA-dependent protein kinase, catalytic subunit (DNA-PKcs). MRN senses and directly binds DSBs and is primarily responsible for the recruitment and activation of ATM[1-4]. KU also binds DSBs and activates the DNA-PKcs kinase, which has roles in the non-homologous end-joining (NHEJ) repair pathway and V(D)J recombination[5, 6]. It is not known why there are two DSB sensors needed in the mammalian DSB response.

To gain mechanistic insight into the early DSB response, we examined cells lacking one or both DNA damage sensors and used pharmacological inhibitors to inhibit the activity of either ATM, DNA-PKcs, or both. We disabled the MRN complex in mouse embryonic fibroblast cells (MEFs) derived from mice that

contained one *Mre11* null allele and one Cre/LoxP conditional allele[7]. Cre recombinase was added to the cells in tissue culture to convert them to *Mre11*$^{-/-}$, which also causes deficiency of the entire MRN complex[7]. Through mouse breeding, we also generated MEFs of MRN genotypes combined with *Ku*-deficiency (*Ku70*$^{-/-}$)[8].

We first set out to investigate how loss of both sensors affects one of the earliest events in the DSB response: phosphorylation of the histone variant H2AX (phosphorylated H2AX is denoted as γH2AX)[9]. After DSB induction, repair proteins are recruited to the sites of damage and can be visualized as punctate dots called foci[10]. Therefore, we analyzed γH2AX foci formation in cells deficient for MRE11, KU70, or both following DNA damage induced by ionizing radiation (IR). Cells were also treated with ATM and DNA-PKcs inhibitors. We found that in control cells, γH2AX foci formation is ATM-dependent. In *Mre11*$^{-/-}$ cells, γH2AX foci formation is dependent on DNA-PKcs. In the absence of KU70, γH2AX foci formation became ATM-dependent. Finally, to our surprise, we observed γH2AX foci formation in the absence of both DNA damage sensors, which was dependent on ATM. This data was also supported by western blot analysis of phosphorylated H2AX. In addition, we found several other ATM-dependent responses restored in the absence of both MRN and KU following DNA damage, including early recruitment of MDC1 to DSBs and the G2-M checkpoint[11, 12]. However, *Mre11/Ku70*-deficiency in mice did not allow for embryonic survival, DNA repair by homologous recombination, or normal IR sensitivity. These findings indicate that

MRN is not absolutely required for the activation of ATM, as previously believed in the DNA repair field.

While our data shows that MRN is not required for initial ATM activation, it might be important for maximizing and sustaining ATM-dependent responses. We noticed that phosphorylation of the ATM substrates KAP1 and SMC1 in MRN/KU-deficient cells was not as robust as in control cells. Furthermore, MDC1 recruitment to DSBs was slightly decreased in cells lacking both DNA damage sensors as compared to control cells. This data would suggest that MRN is needed for maximal ATM activation. This conclusion is further supported by the fact that patients with mutations in *MRE11* (ataxia-telangiectasia-like disorder) have similar clinical phenotypes as to those with mutations in *ATM* (ataxia-telangiectasia)[13, 14]. Furthermore, ataxia-telangiectasia-like disorder (ATLD) patient cells have low levels of the MRN complex, reduced activation of ATM by DSBs, and reduced ATM-dependent phosphorylation of downstream substrates[15].

To gain a better understanding of how MRN and KU function with or without the other in the early DSB response, we analyzed the localization of GFP-tagged NBS1 or KU to DNA DSBs using laser-induced microirradiation and live-cell imaging. We found that MRN was recruited to breaks within 15 to 30 seconds and that KU-deficiency did not affect its recruitment. Moreover, we found that KU was recruited to breaks within 1 second after DNA damage and the absence of MRN did not affect its recruitment. I also examined the formation of KU70 foci in the absence of MRE11 at later time-points following IR treatment[16]. I observed KU70 foci formation within 5 minutes after DNA damage in both control and

MRE11-deficient cells. Control and *Mre11*$^{-/-}$ cells reached the same maximal peak of KU70 foci formation at 5 minutes post-IR. From these experiments, we learned that 1) KU70 is recruited to DSB breaks before MRN and 2) the absence of either sensor does not affect the overall accumulation of the other. These findings suggest that MRN and KU do not compete at DSBs for initial DSB binding. However, it remains unclear as to how they oppose each other at DSBs.

Future Directions

The MRN complex must somehow overcome the rapid binding of KU to DSBs for it to perform its ATM-dependent functions. This leads us to the question of how exactly KU is removed from DNA following the ligation of DSBs. We know that KU is rapidly recruited to DNA breaks before MRN. Studies have also shown in laser micro-irradiation experiments that KU signal steadily depletes within hours after the initial damage, suggesting that KU is somehow being removed from the DNA[17]. KU differs from other DNA repair proteins in that it has a ring-like structure that encircles the DSB, suggesting that once the DSB is ligated, it becomes trapped[18, 19]. One possible mechanism for KU removal is through the protein degradation pathway. A previous study found that poly-ubiquitination of KU80 Lysine 48 leads to its degradation by SKP1-CUL1-FBXL12 (SCF) E3 ubiquitin ligase complex in *Xenopus laevis*[20]. Another intriguing possibility is that the DNA is directly cut, thus allowing for KU to escape. Indeed, one study showed in yeast that MRN performed an endonucleoytic incision to the DNA, followed by digestion of the DNA to allow for KU removal[17]. These are all

intriguing possibilities, but it is important to note that they were performed in different biological systems.

Another question that remains is the mechanism by which ATM is activated. Our data demonstrates that direct interaction between the MRN complex (NBS1 specifically) with ATM is not required for ATM to be activated, which was the previous model in the field[4]. One possibility is that ATM is activated directly by DNA ends. In 1999, Stephen Jackson's group published a report showing that ATM binds directly to DNA[21]. Furthermore, they showed that purified ATM phosphorylates p53 after stimulation by DNA[21]. This data supports the notion that, indeed, ATM can become activated in the absence of MRN. However, these were *in vitro* studies using purified proteins and so additional experiments will need to be conducted to confirm these results in mammalian cells. Another intriguing hypothesis is that ATM becomes activated from by-products of DNA damage. Reactive oxygen species (ROS) are formed during the normal metabolism of oxygen and have important roles in cell signaling and homeostasis[22]. During times of environmental stress, including exposure to ultra-violet light (UV), ROS levels can increase dramatically[23]. Tanya Paull's group demonstrated in 2010 that ATM acts as a redox sensor in addition to its role as a sensor of DSBs[24]. They showed direct activation of ATM by oxidative stress independent of DSBs and the MRN complex. However, they also observed that ROS-dependent activation of ATM did not lead to phosphorylation of H2AX, and so this seems like an unlikely mechanism for our study[24]. Another possibility is that ATM is activated by an unknown mediator

protein in the absence of both MRN and KU. One probable candidate is ataxia-telangiectasia and rad3-related protein (ATR), a kinase activated by the presence of single-stranded DNA[25]. Penny Jeggo's group showed that ATM phosphorylation at Serine 1981 is ATR-dependent and ATM-independent following replication fork stalling or UV treatment[26]. Thus, it is possible that long stretches of single-stranded DNA are being created as intermediates during end-resection, leading to activation of ATM by ATR. To test this hypothesis, we could treat *Mre11*$^{-/-}$*Ku70*$^{-/-}$ cells with an ATR inhibitor and look for activation of ATM and phosphorylation of downstream substrates after DNA damage[27]. Studies using the ATR inhibitor VE-821 have been marred by the fact that increased inhibition of ATR also led to inhibition of ATM and DNA-PKcs. For these reasons, it would be interesting to test the new ATR inhibitor known as VX-970, which is the first selective inhibitor of ATR to be entered into clinical trials[28]. Finally, *in vitro* experiments demonstrated that ATM could be activated in the absence of MRN in *Xenopus* egg extracts, but only when there were high levels of DNA ends present[29]. If ATM can be activated directly by DNA ends, it is possible that MRN and KU allow ATM to access the DNA, and MRN then functions to maximize ATM activation and signaling. Mammalian biology is significantly more complex than the above *in vitro* experiments, and so future experiments will need to test this hypothesis in a cellular model.

Perhaps the most clinically relevant conclusion from our study is that some ATM-dependent responses can be restored in the absence of both MRN and KU. From this data, we can propose a novel therapeutic approach for the

treatment of patients with MRN-deficiency. Patients with mutations in the *MRE11* gene have ATLD, characterized by progressive cerebellar ataxia and a varied predisposition to cancer[13, 15]. Patient cells have low levels of the MRN complex, defects in cell cycle checkpoint activation, hypersensitivity to radiation, and an increase in chromosomal aberrations as compared to controls[15]. While MRN deficiency in mice leads to embryonic lethality, patient mutations are hypomorphic, thus preserving essential functions of the MRN complex for human development[30]. Our findings point to the intriguing possibility that inhibiting KU in ATLD patients could restore ATM-dependent functions (Figure 5.1A). A novel specific inhibitor of the KU70/80 heterodimer was recently created using an *in silico*, pocket-based drug discovery approach[31]. Experiments confirmed KU-inhibitory activity of the small molecule inhibitor in the low micro-molar range, capable of disrupting the binding of the KU heterodimer to DNA substrates and impairing KU-dependent activation of DNA-PKcs[31]. It would be interesting to investigate the use of this small molecule inhibitor in *Mre11*$^{-/-}$ cells to determine if inhibition of KU and subsequent DNA-PKcs activity restores ATM-dependent phosphorylation of H2AX, MDC1 recruitment to DSBs, and the G2/M checkpoint. If so, drug studies can then move onto ATLD mouse models and eventually human patients.

Roles for MRN and CDK2 interaction in S-phase checkpoint control

Summary and Insights

In Chapter III, I investigated the biological functions of MRE11 interaction with cyclin dependent kinase 2 (CDK2). Our lab first described the MRE11-CDK2 interaction in 2012. Buis et al. showed that MRE11 interaction with CDK2 is required for CTIP phosphorylation and interaction with BRCA1, which, in part, regulates HR capacity in normally dividing cells[32]. The MRE11 C-terminus is required for interaction with CDK2, which is absent in an inherited patient allele termed ataxia-telangiectasia-like disorder 1 (ATLD1). These studies demonstrated that functions of the MRN complex are not restricted to DNA damage responses, but include regulating HR capacity during the normal mammalian cell cycle[32].

My thesis work has been focused on further understanding roles for the MRE11-CDK2 interaction in response to DNA DSBs and in cell cycle checkpoint function. We first set out to investigate the consequences of DNA damage induced by IR on the MRE11-CDK2 interaction. We found that IR led to disruption of the MRE11-CDK2 interaction in an ATM-dependent manner. We also found that IR treatment caused a decrease in CDK2 catalytic activity levels in cells at timepoints where we also observed disruption of the MRE11-CDK2 interaction. Previous studies have demonstrated conflicting data of how cells respond to ionizing radiation treatment. One study showed that 10Gy IR caused a decrease in catalytic CDK2 activity in the human osteosarcoma cancer cell line, U2OS, 1-2 hours post-treatment[33]. However, another paper showed that 10Gy caused an

increase in CDK2 activity levels in response to IR up to 10 hours post-treatment in the chicken B cell line DT40[34]. Perhaps responses to inhibition of CDK2 activity after IR depend on cell type or the organism from which they are derived. Future studies will need to investigate if other types of DNA damage (such as aphidicolin-induced replication stress) also disrupt the interaction and if the disruption is dependent on additional DNA damage kinases (i.e. ATR or DNA-PKcs). While we cannot definitively say if the disruption of the interaction after IR caused the decreased CDK2 activity levels, it led us to hypothesize that the MRE11-CDK2 interaction is a component of an S-phase checkpoint pathway.

The intra-s-phase checkpoint is activated to delay progression through S-phase allowing time for DNA repair to occur before mitosis and to prevent the accumulation of genomic damage. Several S-phase checkpoint pathways have been previously described[33, 35-39]. One pathway is thought to be controlled by the ATM signaling machinery, in which checkpoint kinase 2 (CHK2)-mediated degradation of M-phase inducer phosphatase 1 (CDC25A) causes inhibition of CDK2 activity and inhibition of cell division control protein 45 homolog (CDC45) onto chromatin, which in turn, inhibits DNA replication[33, 35]. This data is supported by the fact that cells derived from patients with A-T caused by mutations in the *ATM* gene, exhibit a radioresistant DNA synthesis (RDS) phenotype, in which cells do not inhibit DNA replication in response to IR[40].

Because ATM activation is primarily controlled by the MRN complex, it makes sense that MRE11 could have unknown roles in controlling the S-phase checkpoint. Previous studies have investigated the role of the MRN complex in

RDS. MEF cells derived from *Mre11*$^{ATLD1/ATLD1}$ mice (mice expressing one of two *Mre11* alleles which results in C-terminal truncation of the MRE11 protein) exhibited a severe defect in inhibition of DNA synthesis after IR. These mice were viable, but embryonic viability in homozygous mothers was dramatically reduced[41]. Additionally, primary fibroblast cells derived from patients with mutations in *NBS1* and *MRE11* were found to have an intermediate radioresistant DNA synthesis phenotype[35]. Importantly, loss of any of the components of the MRN complex leads to embryonic lethality in mice[30, 42, 43]. Thus, *MRE11* patient alleles must be caused by hypomorphic mutations that retain functions necessary for embryonic development in humans.

We previously generated a germline mouse allele in which *Mre11* is conditionally deleted, leading to deficiency of the entire MRN complex[30]. We utilized MRE11-deficient MEFs from these mice and show that loss of the MRN complex leads to lower basal levels of CDK2 activity in cells. In response to IR, cells displayed a severe defect in inhibition of catalytic CDK2 activity, comparable to defects caused by ATM-deficiency, and an intermediate RDS phenotype. This data suggests that the MRN complex contributes to inhibition of CDK2 activity after IR. The intermediate RDS phenotype we observed mirrors the results found in human *NBS1* and *MRE11*-mutant patients[33]. This data is surprising, as we expected that loss of the entire MRN complex would generate a complete RDS phenotype, like the one seen in ATM null cells, since MRN-deficiency causes loss of ATM signaling. One possibility is that another kinase, such as ATR or DNA-PKcs, can substitute for ATM in facilitating S-phase checkpoint functions when

MRN is absent. The lower basal levels of CDK2 activity can be explained by the slow proliferation and severe genomic instability characteristic of these cells due to loss of the entire MRN complex[30].

Because ATLD patient cells have low levels of the MRN complex, structure-function studies can be difficult to perform[13, 15]. Thus, we generated an *Mre11*^{ATLD1}-mutant expressing cell line in which the MRE11 C-terminus is truncated and endogenous MRE11 is deleted by the Cre/LoxP system[44]. We have previously demonstrated that MRE11 C-terminal truncation still allows for MRN complex formation and ATM signaling[44]. Thus, any defect found in this mutant can be attributed to loss of the MRE11 C-terminus specifically, and not due to low levels of the MRN complex and subsequent loss of ATM signaling. We found lower basal levels of CDK2 activity in these cells, comparable to MRE11-deficiency. In response to IR, MRE11 C-terminal truncation still allowed for inhibition of CDK2 activity and DNA synthesis. This data would suggest that the S-phase checkpoint defects seen previously in the *Mre11*$^{ATLD1/ATLD1}$ mice and *MRE11* and *NBS*-mutated patients is indeed caused by low levels of the MRN complex and loss of ATM signaling, and not due, specifically, to loss of the MRE11 C-terminus alone.

However, another hypothesis is that what looks like an intact S-phase checkpoint in the MRE11 C-terminal mutant is in fact “constitutively active”. A constitutively active checkpoint in the MRE11 C-terminal mutant would signify that the MRE11 C-terminus is indeed important for S-phase checkpoint function, and that this is independent from ATM-signaling. If the checkpoint were always on, we

would predict that cells would grow slowly and have decreased basal levels of CDK2 activity. Indeed, these are phenotypes we observed in MRE11 C-terminal truncated mutated cells. The lower levels of CDK2 activity caused by loss of the MRE11 C-terminus mimicked the lower levels observed due to loss of the entire MRN complex. Another interpretation of this data is that genetic disruption of the MRE11-CDK2 interaction mimics IR-induced disruption of the interaction, leading to lower levels of CDK2 activity. To further investigate this phenotype, we measured cell proliferation rates and genomic instability in these cells. We observed growth defects in the MRE11 C-terminal truncation mutant comparable to the defects in proliferation caused by MRE11-deficiency. However, we observed only a mild genomic instability phenotype in the MRE11 C-terminal truncation mutant. In contrast, MRE11-deficiency is known to cause serve genomic instability[30]. This data would suggest that the mild genomic instability observed in the MRE11 C-terminal truncation mutant is not sufficient to cause the slow proliferation and low levels of basal CDK2 activity. Additionally, deficiency of MRE11 nuclease activity did not impact CDK2 activity levels.

Together, our data points toward specific functions of the MRE11 C-terminus in controlling CDK2 activity levels that are separate from MRE11 nuclease activity. We conclude from these studies that the MRE11 C-terminus has roles for maintaining normal levels of CDK2 activity, and that it accomplishes this task through its interaction with CDK2. Therefore, the MRE11-CDK2 interaction represents a novel S-phase checkpoint pathway for controlling CDK2 activity in response to DNA damage.

Future Directions

There are many questions left to investigate. We initially observed that disruption of the MRE11-CDK2 interaction after IR treatment is dependent on ATM, but the mechanism remains unclear. One hypothesis is that ATM phosphorylates the C-terminus of MRE11 after DNA damage, and that this phosphorylation event causes MRE11 and CDK2 to dissociate. A recently published paper demonstrated that, indeed, the MRE11 C-terminus has several SQSQ sites, which are ATM phosphorylation motifs, and that ATM phosphorylates these sites after IR[45, 46]. To test this hypothesis, we could mutate the Serines to Alanines within the SQSQ sequences, thus generating unphosphorylatable mutants, and observe the interaction status following IR treatment. It would also be interesting to utilize mass spectrometry to identify additional interaction partners or post-translational modifications of the MRE11 C-terminus.

Furthermore, the mechanism behind the decrease in basal CDK2 activity in the MRE11-mutants remains unclear. Full activation of CDKs requires binding of CDKs to a cyclin and phosphorylation on an activating site. Inhibitory phosphorylation on CDKs inhibit CDK2 activity. We did not observe any difference in phosphorylation of CDK2 on Tyrosine 15, an inhibitory phosphorylation event, in our MRE11-mutant cells that could explain the decrease in basal levels of CDK2 activity. However, we did find that the MRE11-deficient and MRE11 C-terminal truncated mutant cells had lower levels of CDK2 Threonine 160 phosphorylation, which in theory, could reduce CDK2 catalytic activity. There was no difference in Cyclin A binding in these cells. It is known that the activating threonine residue is

located in a loop of amino acids termed the T-loop, which blocks access of ATP to the catalytic domain. Crystal structure studies have shown that binding of a cyclin to CDK causes a conformational change in the CDK which makes it more accessible for the activating phosphorylation. Therefore, it would be interesting in the future to perform structural studies to examine how loss of the MRE11 C-terminus affects the structure of the MRE11 protein. Perhaps the C-terminus is important for allowing the T-loop to become accessible for activating phosphorylation and loss of the MRE11 C-terminus results in a blocked catalytic domain, resulting in reduced phosphorylation of Threonine 160 and decreased CDK2 catalytic activity. Additionally, I would be curious to know how ionizing radiation affects the structures of both MRE11 and CDK2. It is possible that ionizing radiation also causes a conformational change in one of these proteins, leading to dissociation of the interaction.

Buis et al. determined that the MRE11 C-terminus is essential for interaction with CDK2, and that disruption of this portion of the protein leads to a significant decrease in phosphorylation of the CDK2 substrate CTIP[32]. Other substrates of CDK2 have recently been identified, including NBS1, C-MYC, and RAG2[47-50]. It has been shown that CDK2 phosphorylates NBS1 on Serine 432 and that this phosphorylation is important for protecting cells from IR-induced cell death[51]. This data demonstrates a specific requirement for the catalytic activity of CDK2 in the survival of human cells during the DNA damage response. Another study found that this modification stimulates MRN-dependent conversion of DSBs into structures that are substrates for repair by HR[47]. In future studies, it would be

intriguing to determine if CDK2-dependent phosphorylation of NBS1 occurs in the absence of the MRE11 C-terminus. If not, this data would suggest that the MRE11 C-terminus, through interaction with CDK2, is required for CDK2-dependent phosphorylation of NBS1 to facilitate the protection of cells from IR and for the stimulation of DSB repair by HR. Moreover, a defect in CDK2-dependent phosphorylation of NBS1 could, in part, explain the lower levels of CDK2 activity caused by loss of the MRE11 C-terminus.

CDK2 has also been found to have roles in regulating V(D)J recombination through CDK2-dependent phosphorylation of RAG2 on Threonine 490. Mutation of this site abolishes degradation of RAG2 protein, which results in aberrant recombination events during V(D)J recombination[49]. The cell cycle dependence of V(D)J recombination suggests it may be interesting in future studies to determine how genetic disruption of the MRE11-CDK2 interaction affects lymphogenesis and V(D)J recombination in mice.

Interestingly, hypomorphic mutations in the MRN complex cause a variety of clinical phenotypes in human patients, including cancer predisposition. Individuals with NBS exhibit a predisposition to cancer, immunodeficiency, and microcephaly[15]. A predisposition for cancer in ATLD is variable, but two Japanese brothers with ATLD recently died of pulmonary adenocarcinoma[52]. In addition, the *Mre11*^{ATLD1ATLD1} mouse was not prone to malignancy, suggesting that the severe checkpoint failure and chromosomal instability observed in the mouse were insufficient to enhance the initiation of tumorigenesis[41]. To determine roles for the MRE11 C-terminus in tumorigenesis, it would be interesting to delete this

region of the MRE11 protein in mice and evaluate them for tumor formation. Additionally, the MRE11 C-terminus could be specifically deleted in B-lymphocytes and the mice monitored for lymphoid tumors. By deleting the MRE11 C-terminus, any results would be attributed to that region of the protein specifically, and not due to low levels of the MRN complex and loss of ATM signaling as in the *Mre11*$^{ATLD1/ATLD1}$ mouse model.

We have preliminary data suggesting that the MRE11-CDK2 interaction is not disrupted after IR in two human cancer cells lines: U2OS and HeLa. We hypothesize that disruption of the MRE11-CDK2 after DNA damage is important for initiating the S-phase checkpoint by lowering CDK2 activity levels. If this is true, one might expect that the interaction cannot be disrupted in cancer cells, thus promoting CDK2 activity and uncontrolled cell division. Indeed, our cancer cell data supports this hypothesis. It will be important in future studies to investigate the status of the MRE11-CDK2 interaction in additional human cancer cell lines. Furthermore, if disruption of the MRE11-CDK2 interaction is truly important for regulating the S-phase checkpoint, a small molecule inhibitor for this interaction could be a useful therapeutic tool in the treatment of cancer. Synthetically disrupting the interaction in cancer cells would theoretically restore S-phase checkpoint functions, allowing for DNA repair to occur and thus preventing genomic instability and tumorigenesis (Figure 5.1B).

Roles for MRN in tumorigenesis

Summary and Insights

In Chapter IV, I sought to further understand roles for the MRN complex in the initiation and progression of cancer. It is known that mutations in DNA repair and cell cycle checkpoint genes can lead to inherited syndromes associated with a predisposition to cancer. For example, inherited deficiencies in *ATM* lead to the disease ataxia-telangiectasia (A-T), which presents with clinical features such as immunodeficiency, sensitivity to ionizing radiation, and lymphomas[14, 53]. Likewise, hypomorphic mutations in *MRE11* cause the disease ataxia-telangiectasia-like disorder (ATLD), which is characterized by cerebellar ataxia and a variable predisposition to cancer[15]. Hypomorphic mutations in *NBS1* lead to Nijmegen breakage syndrome in which patients present with a severe predisposition to cancer, immunodeficiency, and microcephaly[54]. While mice lacking components of the MRN complex do not survive, no known human alleles are null, suggesting that human patients have partial loss-of-function mutations which still allow for functions of the MRN complex necessary for human development[42, 43, 55]. The cancer-prone phenotypes linked with MRN-associated diseases suggest MRN functions as a tumor suppressor and is necessary for the prevention of cancer development.

To further investigate how MRN functions in cancer, we examined the impact of MRE11-deficiency and MRE11 nuclease deficiency in B-lymphocytes in engineered mouse models in our lab. We utilized CD19-cre conditional alleles,

which express cre recombinase specifically in B cells, to delete MRE11 in lymphocytes[56]. We also crossed mice to germline *p53* knockout mice[57]. Surprisingly, the MRE11 deficiencies did not predispose mice to lymphomagenesis. Some of the mice had thymic lymphomas of T-cell origin, a common outcome of p53 deficiency[58]. Additionally, some of the mice had nonlymphoid tumors. MRE11/p53-deficient B cells were also observed to possess oncogenic chromosomal translocations, including translocations between the immunoglobulin heavy locus (IgH) and the proto-oncogene MYC. The observation that mice harboring MRE11-deficient B cells did not succumb to lymphogenesis would suggest that MRE11, in some capacity, is required for oncogenesis. This would further refute the notion that MRE11 is a classic tumor suppressor.

To further access the roles of MRE11 in tumorigenesis, we examined the impact of MRE11 deficiency and MRE11 nuclease deficiency in *Artemis/p53* null mice, which are strongly prone to the development of lymphomas[59]. Again, to our surprise, we observed that MRE11 deficient mice in these cohorts primarily developed thymic tumors and no mice developed pro-B lymphomas. This data would suggest that MRE11 mutation suppresses pro-B lymphoma development in a mouse model in which they normally arise[59, 60]. Additionally, chromosomal translocations were observed in MRE11 and MRE11/ARTEMIS-deficient fibroblasts, implying that chromosomal translocations can be generated in the absence of MRE11. The lack of tumors in these mice is unexpected given that several other DNA repair mouse models, including *DNA Ligase IV, Xrcc4, and Artemis*, which, when combined with *p53* deficiency, predominantly predispose the

mice to B-cell lymphomas characterized by IgH:MYC chromosomal translocations[59-62]. However, we cannot rule out the possibility that we were not able to detect B-cell lymphomas before thymic tumor development in the MRE11/p53-deficient mice.

The MYC proto-oncogene was first discovered for its role in retrovirally-mediated tumorigenesis and later for its activation and overexpression through chromosomal translocations in Burkitt's lymphoma[63, 64]. In normal biology, MYC protein is a transcription factor that has roles in DNA replication, cell proliferation, and cell growth[65]. During DNA replication, CDC45 is loaded onto the pre-replication complex and this process initiates DNA replication in S-phase, which is also controlled by the activity of CDKs[66]. C-MYC has been found to directly interact with CDC45, thus stabilizing components of the pre-replication complex[67, 68]. MYC overexpression, on the other hand, deregulates S-phase, resulting in replication stress and genomic instability[65, 69-71]. In some cases, MYC causes normal cells to grow and replicate DNA, but these cells cannot divide and become polyploid. Additionally, MYC overexpression can enforce replication in a manner that results in DSBs, by either blocking DNA repair or increasing oxidative stress, causing DNA damage[70]. With this knowledge, we hypothesized that MRE11 may promote the survival of cells harboring oncogenic translocations and investigated the impact of MYC overexpression and MRE11 deficiency on cell survival. Interestingly, we found that inhibition of MRE11 nuclease activity, using the inhibitor Mirin, selectively increased DNA damage and apoptosis in U2OS cells overexpressing the C-MYC oncogene, suggesting that C-MYC overexpressing

cells rely on MRE11 nuclease activity for survival. Therefore, MRE11 is not a classic tumor suppressor. Together, our study supports the conclusion that MRE11 nuclease activity may be required for the progression of cancer and that inhibiting the nuclease activity of MRE11 could serve as a promising target in C-MYC-driven cancer.

Future Directions

In future studies, it would be interesting to delete MRE11 specifically in the T-cell lineage and examine mice for the development of thymic tumors. While lymphomas are frequently characterized by MYC overexpression and IgH:MYC translocations, thymic tumors typically are not[72]. Therefore, if the mice succumb to thymic tumors, this data would suggest that MRE11 specifically functions in MYC-driven cancers. On the other hand, an absence of thymic tumors would suggest that MRE11 functions in a wider range of cancers not specifically characterized by MYC overexpression.

While we primarily focused our attention on the consequences of C-MYC overexpression in oncogenesis, N-MYC amplification is associated with cancers as well, including retinoblastoma and neuroblastoma[73, 74]. Importantly, the amplification of N-MYC is found in approximately 25% of neuroblastoma cases and correlates with high-risk disease and prognosis[75]. Amplification of N-MYC currently represents the best characterized genetic marker in neuroblastoma[75]. Because N-MYC has roles in cell proliferation, growth, and apoptosis, similarly to C-MYC, it would be interesting to utilize patient neuroblastoma cells that

overexpress N-MYC in our studies to test the effect of MRE11 nuclease inhibition on cell survival. Indeed, Petroni et al. showed in 2016 that the MRN complex is required for N-MYC-dependent proliferation in N-MYC overexpressing cells and that inhibition of MRN resulted in DNA damage, activation of the DNA damage response, and cell death[76, 77]. Thus, studies using Mirin could provide evidence of a therapeutic role for MRE11 nuclease activity in N-MYC driven neuroblastoma.

Additionally, it would also be intriguing to investigate the development of tumors in the well-characterized E mu-myc transgenic mouse model, which has a high incidence for spontaneous lymphoma and leukemia in B cells, upon treatment with an MRE11 nuclease inhibitor[78]. If our hypothesis is correct, I would predict a decrease in size and number of B cell tumors as compared to untreated controls. Likewise, we could utilize patient-derived Burkitt Lymphoma B cells, which have IgH:MYC translocations and exhibit overexpression of C-MYC, to test the efficacy of MRE11 nuclease inhibitors on development of colonies in a colony forming assay[64]. Anchorage-independent growth, a hallmark of cancer, is the ability of transformed cells to grow independently of a solid surface. The soft agar colony formation assay is a well-established method for characterizing this capability *in vitro* and is one of the most stringent tests for malignant transformation in cells[79]. Therefore, I would predict treatment with an MRE11 nuclease inhibitor to prevent colony formation of Burkitt Lymphoma B cells.

We have previously shown that MRE11 interacts with CDK2 and that this interaction has roles for regulating homologous recombination in normally dividing

cells[32]. Additionally, the MRE11 C-terminus is required for interaction with CDK2 and is absent in an ATLD patient allele[32]. Recently, C-MYC was identified as a substrate of CDK2. Several reports have demonstrated that CDK2 phosphorylates C-MYC on Serine 62 and that this phosphorylation has a role in suppressing oncogene and replication stress-induced senescence[50, 80]. This data suggests that CDK2 could potentially be used as a therapeutic target in C-MYC-driven cancers. In future studies, it would be interesting to determine if MRE11, through interaction with CDK2, is required for CDK2-dependent phosphorylation of C-MYC and in suppression of oncogene-induced senescence. To test this hypothesis, we could overexpress C-MYC in an MRE11 C-terminal truncation mutant (in which the MRE11-CDK2 interaction is genetically disrupted) and measure cell proliferation. If the MRE11 C-terminus is important for suppression of oncogene-induced senescence, I would expect that overexpression of C-MYC would selectively inhibit proliferation in the MRE11 C-terminal truncation mutant as compared to controls.

Although the inherited hypomorphic mutations of the MRN complex in humans lead to cancer predisposition, complete loss of the MRN complex in mice is not compatible with malignancy, as these mice are not viable[30, 42, 43]. This suggests that hypomorphic mutations of MRN retain functions needed for tumorigenesis (and embryonic development). We hypothesize that the nuclease activity of MRE11 is one of the functions that supports cancer development. This notion is supported by the fact that no human inherited MRN mutations disrupt the nuclease activities of MRE11. For example, the patient allele *MRE11*^{ATLD1} is

caused by loss of the MRE11 C-terminus[15]. Patients with *MRE11*^{ATLD1} present with varied predisposition to cancer, but some patients have been reported to have breast cancer[81]. This mutation likely maintains sufficient nuclease activity for tumorigenesis given that it still allows for organism survival and the N-terminal nuclease domain maintains its activity and structure even in the absence of the C-terminus.

Defects in the DNA damage response due to oncogenic activation or tumor suppressor inactivation lead to replication stress, a phenomenon unique to cancer cells in which DNA replication persists to meet the demands of unrestrained cell proliferation, despite the presence of unrepaired DNA lesions[82, 83]. Thus, replication stress causes genomic instability and further potentiates oncogenic transformation. In normal cells, replication stress can be countered by the DNA damage response. However, in cancer cells where the DNA damage response is defective, high levels of replication stress may induce cancer cell death and counteract cancer progression[83, 84]. Thus, further enhancing replication stress may provide a promising alternative to treat cancer[85]. Indeed, our studies demonstrate that pharmacologic inhibition of MRE11 nuclease activity by the small molecular inhibitor, Mirin, selectively decreases survival of MYC overexpressing cells (Figure 5.1C)[86]. Further studies will need to be conducted, especially in mice, before Mirin or other MRE11 nuclease inhibitors become viable options for clinical trials in humans. Other drugs using this “synthetic lethality” approach are already FDA approved, including the PARP1 inhibitor, Olaparib[87]. Several other drugs are in in Phase I or II trials, including drugs that target the DNA repair

proteins checkpoint kinase 1 (CHK1) and ATR[88, 89]. It will be important to investigate how these drugs can be used in combination with other therapies and possible mechanisms of drug resistance. In conclusion, increased understanding of oncogene-induced DNA replication will greatly contribute to the development of novel cancer therapies.

Concluding Remarks

In summary, my thesis work has contributed to advances in our understanding of the MRN complex in DNA damage responses and signaling, cell cycle checkpoint control, and in oncogenesis. Through my work, I discovered that MRN is not absolutely required for activation of ATM, that the MRE11 C-terminus has roles in controlling normal levels of CDK2 activity through direct interaction with CDK2, and that MRE11 nuclease activity is a potential therapeutic target in MYC-driven cancers (Figure 5.2).

An important goal of studying the MRN complex has always been to uncover mechanisms underlying cancer initiation and progression that can eventually lead toward development of clinical therapies. My studies have revealed that the biology of the MRN complex in relation to cancer is more complicated than previously thought. It appears that this DNA repair complex has both pro- and anti-tumor capabilities. Roles for the MRN complex in preventing cancer are evident by the fact that patients who have hypomorphic mutations in members of the complex are predisposed to cancers. Additionally, MRN's functions in facilitating DNA repair and cell cycle checkpoints are central for persevering the integrity of the genome. My work also shows that MRN has roles in controlling CDK2 activity important for regulation of S-phase checkpoint functions. This data, together, supports the notion that MRN is a tumor suppressor. On the other hand, we show that B-cell specific deficiency of MRN or MRE11 nuclease activity does not predispose mice to lymphomas and prevented lymphogenesis in another mouse model prone to these tumors. We also show

that inhibition of MRE11 nuclease activity selectively kills cells overexpressing the oncogene C-MYC. These studies suggest that some functions of the MRN complex, such as MRE11 nuclease activity, promote oncogenesis. It is surprising and perplexing that the MRN complex can have functions that both suppress and promote oncogenesis and future studies will need to further unravel these mysteries, especially in relation to the development of anti-cancer drugs that target this complex. There are many questions that remain unanswered, but I hope my work contributes toward future avenues of investigation in the lab with the goal of one day positively impacting patients who are fighting cancer.

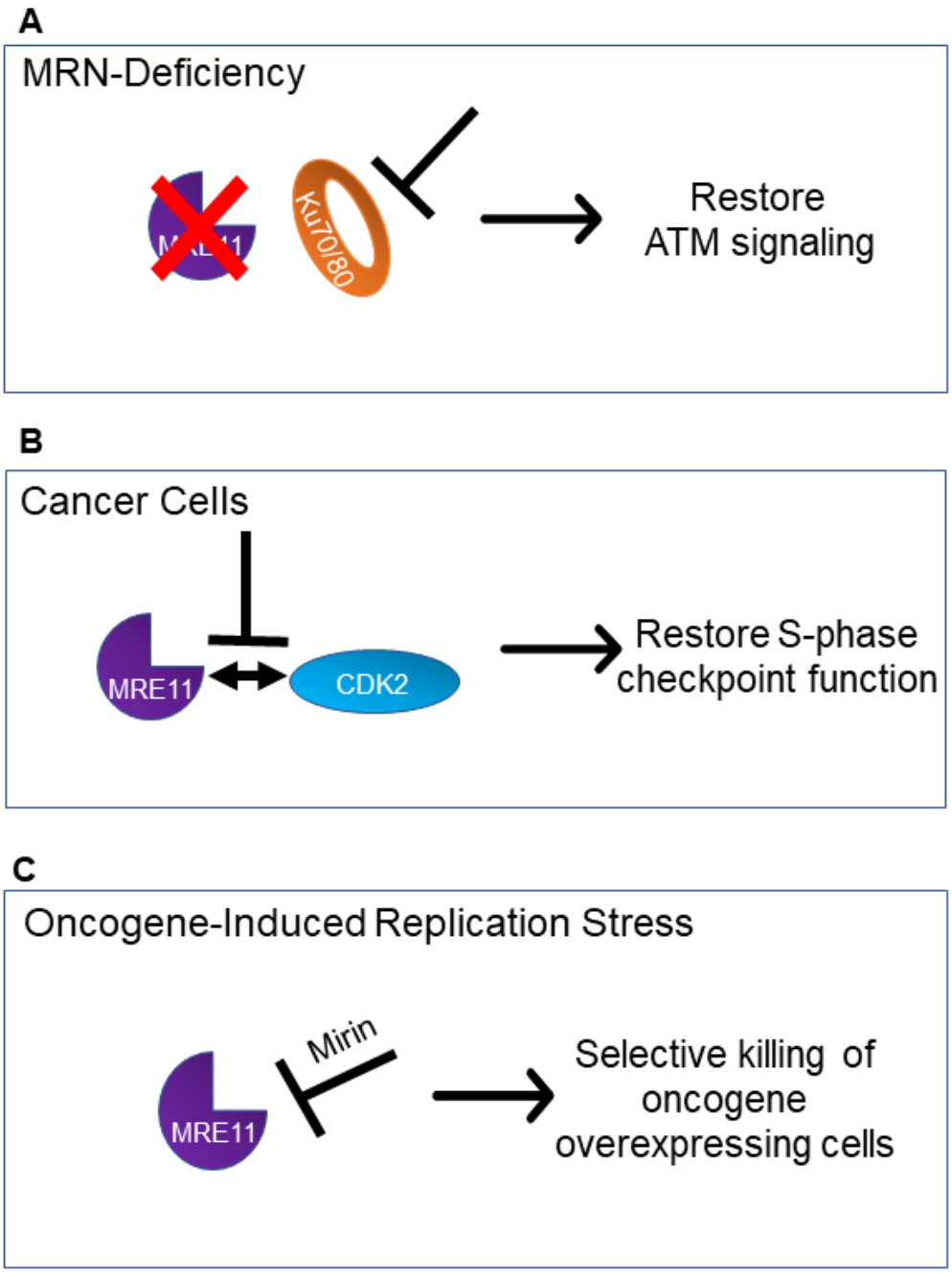
A
MRN-Deficiency
Ku70/80
Restore
ATM signaling
B
Cancer Cells
MRE11
CDK2
Restore S-phase
checkpoint function
C
Oncogene-Induced Replication Stress
Mirin
MRE11
Selective killing of
oncogene
overexpressing cells

Figure 5.1. Model for therapeutically targeting the MRN complex. (A) We demonstrate in our studies that ATM activation and signaling is restored in the absence of both DNA damage sensors, MRN and the KU70/KU80 heterodimer. Based on these findings, we hypothesize that inhibiting KU in ATLD patients could restore ATM-dependent functions. **(B)** We demonstrate that disruption of the MRE11-CDK2 interaction after DNA damage is important for inhibiting CDK2 activity and initiating an S-phase checkpoint pathway in normal cells. However, we found that two cancer cell lines did not exhibit disruption of the interaction after DNA damage. Therefore, we hypothesize that synthetically inhibiting the MRE11-CDK2 interaction in cancer cells with a small molecule inhibitor could restore S-phase checkpoint functions, thus preventing genomic instability and tumorigenesis. **(C)** We demonstrate in our studies that pharmacologic inhibition of MRE11 nuclease activity with the small molecule inhibitor, Mirin, selectively decreased the survival of MYC overexpressing cells. Therefore, we hypothesize that inhibiting MRE11 nuclease activity could be a promising therapeutic strategy in cancers with oncogene-induced replication stress.

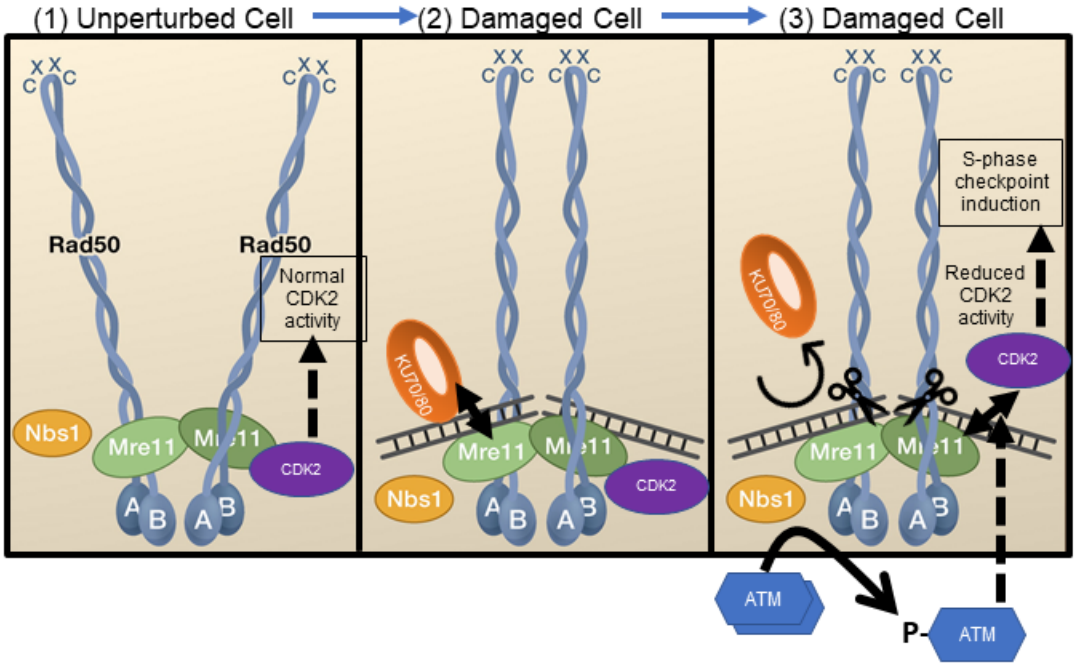

Figure 5.2. Model for summary of thesis work. In unperturbed cells, MRE11 interaction with CDK2 promotes normal CDK2 activity and cellular proliferation. Upon DSB induction, MRN opposes KU to bind DSBs first so that MRN can facilitate activation of ATM. Additionally, we demonstrate that direct interaction between NBS1 and ATM is not required for activation of ATM. We next show that irradiation induces ATM-dependent disruption of the MRE11-CDK2 interaction and causes a reduction in CDK2 activity levels, which we hypothesize is part of an S-phase checkpoint pathway. Finally, we found that MRE11 nuclease activity is important for the survival of cells overexpressing oncogenes, and that a small molecular inhibitor of MRE11 can induce selective killing of MYC-overexpressing cells. My thesis work represents a significant advance in our understanding of MRN in roles for DNA damage kinase activation, regulation of the S-phase checkpoint, and in MYC-driven cancer. Adapted from Kanaar and Wyman, Cell, 2008 and from Buis et al., NSMB, 2012.

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